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# DISSERTATION

Titel der Dissertation

## Minicircle DNA Immobilization in Bacterial Ghosts (BGs): Investigation for the Reduction of Un-recombined Mother Plasmid and Miniplasmid DNA in BGs

Verfasser

Muhammad Abbas

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## Outline of the Thesis

The main goals of this work has been to investigate and develop a system for production of minicircle DNA (mcDNA) loaded Bacterial Ghosts (BGs), that are free of miniplasmid DNA (mpDNA) and un-recombined mother plasmid DNA (mopDNA) through enzymatic digestion of target DNA. Moreover, the purpose has been to develop an efficient method for quantification of anchored mcDNA as well as the amount of residual mpDNA in BGs that is free of any further assumptions.

This Thesis already includes two publications covering topics related to BGs production and its application in DNA vaccine delivery. The following results section is divided into four chapters and an appendix.

- Chapter I: "Investigations reducing un-recombined mother plasmid DNA and miniplasmid DNA in Bacterial Ghosts" discusses different strategies for cloning of a homing endonuclease I-*Tev*II gene on an improved version of Self Immobilizing Plasmid (SIP) which was planned to be used as a tool in reduction of mpDNA in BGs.

- Chapter II: "Minicircle DNA loaded Bacterial Ghosts devoid of un-recombined mother plasmid and miniplasmid DNA sequences by nuclease activation" deals with an alternative approach for reduction of mpDNA and un-recombined mopDNA in BGs involving *Staphylococcus aureus* nuclease A (SNUC) activation.

- Chapter III: "Production of minicircle DNA loaded Bacterial Ghosts carrying a reporter gene and its quantification using quantitative Real Time PCR (qPCR)" is more focused on developing a new strategy for production and quantification of reporter gene encoded mcDNA loaded BGs through qPCR that is different from standard methods used in the past which for quantification of mcDNA was dependent on assumptions.

- Chapter IV: "Improvement of Self Immobilization Plasmids (SIP) used in production of minicircle DNA loaded Bacterial Ghosts" encompasses ways for improvement of SIP to be used in production of BGs harboring mcDNA vaccine through *E* lysis process.

The "Appendix" includes the methods for removal or possible ways to dismantle mutated mobilization (MobM) sequence present on lysis plasmid pGLysivb. It also includes supplementary data for real time PCR quantification of mcDNA.

The publication titled “The bacterial ghost platform system - Production and applications” is published in Bioengineered Bugs (2010). It explains the basic concept of Bacterial Ghost system and covers the topics related to its production and BGs use in white biotechnology.

The manuscript accepted for publication in “Expert Review of Vaccine” titled “Bacterial Ghosts as carriers of protein subunit and DNA encoded antigens for vaccine applications” is more concentrated towards applications of BGs including use of empty BG envelope as a vaccine candidate, BGs as a carrier of DNA /protein antigens, their possible uses in human and veterinary medicine, and their use as an immunocontraceptive drug delivery platform.

Each publication and all chapters have their own introduction, material and methods, results and discussion referring to the topic in consideration. This version of describing the results obtained should facilitate an easier conversion of chapters into manuscripts for publication as the standard form of description in one block.

# OBJECTIVES AND AIMS OF THIS STUDY

Plasmid DNA Vaccination is fast and emerging technique, which is being investigated in ongoing clinical trials. Its ability to elicit both humoral and cellular immunity is key to its wide spread use in basic and advanced research. However, its safety concerns and absence of efficient delivery systems are the key limitations, which restrict its use. The issues have been addressed separately.

The safety issue has been addressed by the introduction of small DNA molecules known as minicircle DNA (mcDNA) which lack bacterial backbone (BB) sequences and antibiotic resistance cassettes. Similarly, the issue of absence of efficient delivery systems has been addressed through the introduction of Bacterial Ghosts (BGs), which is used for efficient delivery of mcDNA to increase its availability to the immune cells. Previously these two systems were combined to produce mcDNA loaded BGs however due to presence of miniplasmid DNA (mpDNA) (a by-product of site specific ParA recombination) in BGs its use in clinical trials is still restricted.

The primary objective of this thesis was to investigate and develop a platform for production of mcDNA loaded BGs that are free of mpDNA and any unwanted bacterial sequences by enzymatic digestion of mpDNA preferentially through action of I-*Tev*II a homing endonuclease. I-*Tev*II belongs to group 1 intron of *sunY* bacteriophage T4 which cleaves its target sequence 13-15nt downstream of its intron insertion sites. In the strategy developed to improve the mcDNA system with BGs as carriers the I-*Tev*II gene should be cloned on an advanced version of the Self Immobilizing Plasmid (SIP) in such a way that it is transferred onto the mpDNA upon recombination of mother plasmid DNA (mopDNA). After recombination I-*Tev*II gene should come under direct control of pBAD promoter resulting in its expression and release of enzyme which cleaves the 31bp recognition sequence placed only on mpDNA/un-recombined mopDNA.

Second strategy of reducing the amount of residual DNA sequences in the BG preparation, is based on exploring the suicidal activity of Staphylococcal nuclease A (SNUC). Due to small size of mcDNA and its tight interaction with its specific membrane anchored DNA binding protein (LacI-L'), it is assumed that mcDNA can escape the suicidal activity of SNUC, which

will be activated only after the completion of recombination and lysis process by addition of $CaCl_2$ and $MgCl_2$. Previously SNUC was used for production of gene free BGs, however, it was never combined together with ParA recombination and gene *E* mediated lysis of bacterial cells.

Another objective of this thesis was to develop a technique using quantitative real time PCR (qPCR) that is independent of any further assumptions in quantifying the amount of mcDNA and mpDNA for efficient quantification of *in vivo* loaded mcDNA inside BGs and the amount of un-recombined mopDNA and retained mpDNA in its preparations. Previous approach towards mcDNA quantification had some limitations as the amount quantified had to be deducted from the amount of un-recombined mopDNA, which was an estimate based on densitometric analysis of end recombination product.

BGs can activate both arms of immune response i.e. innate and adaptive immunity. In principle both the systems will lead to in-vivo loading and production of mcDNA inside the BGs that are more likely free of mpDNA/un-recombined mopDNA, eliminating any further need of purification. The development of one step quantification method for detection of mcDNA loaded BGs based on real time PCR technique will therefore serve as quality criterion and revolutionize the mcDNA/mpDNA quantification method.

## Summary of results

This thesis "Minicircle DNA Immobilization in the Bacterial Ghosts: Investigation for the Reduction of Un-recombined Mother Plasmid DNA and Miniplasmid DNA in BGs" discusses the different possibilities for the improvement of current technique for production of minicircle DNA loaded BGs, that are free of residual miniplasmid DNA / un-recombined mother plasmid DNA. Specific changes in the Self Immobilizing Plasmid and use of staphylococcal nuclease A lead to the production of mcDNA loaded BGs that are almost free of miniplasmid DNA / un-recombined mother plasmid DNA. Introduction of new method for detection of plasmid DNA present in BGs in different forms makes the quantification process more easy, efficient and reliable. The study undertaken in this thesis can be best summarized under six themes mentioned below.

**The bacterial ghost platform system-Production and applications**

The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology. BGs are cell envelopes derived from Gram-negative bacteria. BGs are devoid of all cytoplasmic content yet with preserved morphology. BGs exhibit intrinsic adjuvant properties and trigger an enhanced humoral and cellular immune response to the target antigen. BGs are produced by batch fermentation with subsequent product recovery and purification via tangential flow filtration. For safety reasons all residual bacterial DNA is inactivated during the BG production process by the use of staphylococcal nuclease A and/or the treatment with β-propiolactone. The broad spectrum of BGs possible applications in combination with the comparably low production costs make the BG platform technology a safe and sophisticated product for the targeted delivery of vaccines and active agents as well as carrier of immobilized enzymes for applications in white biotechnology.

**Investigations reducing un-recombined mother plasmid DNA and miniplasmid DNA in Bacterial Ghosts**

I-*Tev*II (homing endonuclease) mediated enzymatic digestion of miniplasmid DNA (mpDNA) / un-recombined mother plasmid DNA (mopDNA) in Bacterial Ghosts (BGs) is the basic aim in this particular study. I-*Tev*II gene has been commercially synthesized and

tried so as to clone on advanced version of Self Immobilization plasmid (SIP). Different bacterial strains with tight internal control of expression through $lacI^q$ and several expression vectors have been investigated for possible cloning of this potentially toxic gene. The cloning strategy although trying several alternative pathways has not been entirely successful but it resulted in a new self immobilization plasmid and several other intermediates. Several tries of cloning I-*Tev*II gene has resulted in SIP plasmids carrying mutated I-*Tev*II sequences. Among them there is a clone carrying a deletion mutation of 48bp which is not a frameshift. This deletion of 48bp will lead to shorter protein sequence, however (largely due to time limitations) its functioning has not been tested in final experiment. Little available knowledge, about the working of I-TevII gene has made it difficult for us to understand the underlying reasons for this phenomenon it is therefore assumed that the I-*Tev*II gene in combination with unknown SIP backbone sequences is lethal for the recipient bacteria, and could therefore has not been achieved with the strategy applied. However the resulted SIP plasmid p4a which has better features than its precedent and several other intermediate plasmids can be used in further studies.

**Minicircle DNA loaded Bacterial Ghosts devoid of un-recombined mother plasmid and miniplasmid DNA sequences by nuclease activation**

A new technique based on nuclease activation for the reduction of mpDNA in *E. coli* Nissle 1917 BGs is presented here. The plasmid p4aEYFP-C1 is used to produce mcDNA carrying an enhanced yellow fluorescent protein (EYFP) under eukaryotic expressional promoter, through site specific ParA recombination. The mpDNA and un-recombined mopDNA has been hydrolyzed through the enzymatic activity of *Staphylococcus aureus* nuclease A (SNUC). It has shown through real time quantitative PCR that 2.38% of mcDNA (23-59 plasmid copies / BG) escaped the hydrolysis activity of SNUC. SNUC is more active on fragments of DNA that are larger and easily accessible, i.e. mpDNA and un-recombined mopDNA therefore, using this method the amount of mpDNA in BGs are reduced to 99.48% whereas the remaining amount of mpDNA corresponds to 8.27 plasmid copies / BG, which is much lower than the amount of mpDNA obtained through normal ParA and gene *E* mediated lysis procedure i.e. ~412 plasmid copies/BG. BGs with mcDNA produced through this technique have been tested in macrophages RAW-264.7 cells which lead to the expression of anchored gene.

**Production of minicircle DNA loaded Bacterial Ghosts carrying a reporter gene and its quantification using quantitative real time PCR (qPCR)**

Three different reporter genes, enhanced yellow fluorescent protein (EYFP), red fluorescent protein (RFP) and mutated green fluorescent protein (VenusA206K) were cloned on advanced version of SIP plasmids. These newly cloned plasmids when tested in CCL-20.2 cells resulted in better fluorescence signals as compared to their origin plasmids. Furthermore, these SIP plasmids showed 99.73% recombination efficiency which has been calculated through newly developed quantitative real time PCR (qPCR) method. The newly designed primers presented in this study have the added ability to generate different sized PCR products depending on the form of mopDNA used i.e. (recombined or un-recombined) and through final melt curve analysis of the qPCR product the source of the quantified PCR product can be traced. Through introduction of this new technique, mpDNA and mcDNA is quantified efficiently without the involvement of any further assumptions. This system has another advantage over old quantification method due to its ability to calculate the recombination efficiency without the densitometric analysis of recombination product.

**Improvement of Self Immobilization Plasmids used in production of minicircle DNA loaded Bacterial Ghosts**

As is known, in order to use mcDNA for vaccination purpose it is important to get rid of all unnecessary sequences and it is more desirable to use only those antibiotics that are not commonly used in medical practice. In my research, changes have been made in the structure of SIP plasmid by positioning the resolution site1 (res1) the size of mcDNA has been reduced to only LacOs sequences and multiple cloning site where one can introduce gene of interest where as by inverting the expression cassette LacI-L'/ParA it is now easy to clone genes that can lead to enzymatic digestion of mpDNA after recombination. In this new version of SIP the antibiotic resistance cassette is changed from ampicillin to kanamycin which is favored by the regulatory agencies like Food and Drug Administration (FDA) and European Medicine Agency (EMA).

**Bacterial Ghosts as carriers of protein subunit and DNA encoded antigens for vaccine applications**

Bacterial Ghosts (BGs) represent vaccine delivery systems gifted with outstanding natural adjuvant properties. BGs are empty cell envelopes of Gram-negative bacteria lacking cytoplasmic content yet retaining unaltered all morphological and structural features of their living counterparts. BGs intact surface make-up is easily recognized by professional antigen-presenting cells through pattern recognition receptors making them ideal for mucosal administration through oral, ocular, intranasal or aerogenic routes, which represent the most desirable ways of application in advanced vaccine use. BGs have been designed to be used as carrier of active substances and foreign antigens (protein and/or DNA) for vaccine development. This review highlights the salient features of the BGs versatile multipurpose vaccine platform for application in a wide range of human and veterinary medicines.

# ZIELSETZUNG DER VORLIEGENDEN STUDIE

Vakzinierung mittels DNA Plasmiden ist eine neu aufkommende Technologie, die derzeit in klinischen Studien getestet wird. Dabei stellt die Möglichkeit, sowohl humorale als auch zelluläre Immunität hervorzurufen, den Schlüssel für die Anwendung in Grundlagen- und angewandter Forschung dar. Sicherheitsbedenken und der Mangel an effizienten Trägersystemen gehören allerdings zu den limitierenden Faktoren, die ihre Anwendung bis jetzt beschränken. Diese beiden Aspekte wurden separat behandelt.

Um etwaigen Sicherheitsbedenken entgegen zu wirken, wurden kleine DNA Moleküle, auch bekannt als minicircle DNA (mcDNA), konstruiert; dieser mcDNA fehlt es sowohl an bakteriellen Backbone (BB) Sequenzen als auch an Antibiotikaresistenzkassetten. Die Frage nach effizienten Trägersystemen wurde durch die Verwendung von Bacterial Ghosts (BGs) beantwortet, um Immunzellen mcDNA mit hoher Effizienz zu liefern. Diese beiden Systeme wurden bereits früher kombiniert um mcDNA-beladene BGs herzustellen; wegen des Vorhandenseins von miniplasmid DNA (mpDNA) – einem Nebenprodukt der sequenzspezifischen Rekombination durch parA – war die Verwendung dieser BGs in klinischen Versuchen bisher nicht möglich.

Das Hauptziel der vorliegenden Arbeit war die Untersuchung und Entwicklung einer geeigneten Plattform zur Herstellung mcDNA-beladener BGs, die keine unerwünschte mpDNA oder sonstige bakterielle Sequenzen mehr enthalten. Dies sollte durch den enzymatischen Verdau von mpDNA, bevorzugt durch die Homing Endonuclease I-*Tev*II, gewährleistet werden. I-*Tev*II gehört zum Gruppe 1 *sunY* Intron von Bakteriophage T4 und schneidet seine Zielsequenz 13-15nt downstream der eigenen Intronsinsertionssequenz.

In der hier entwickelten Strategie zur Verbesserung des Systems der BGs als Träger von mcDNA wird das I-*Tev*II-Gen so in eine weiterentwickelte Variante des Selbstimmobilisierenden Plasmids (SIP) kloniert, dass es nach der Rekombination mit der Mutterplasmid-DNA (mother plasmid – mopDNA) auf die mpDNA transferiert wird. Nach erfolgter Rekombination gerät das I-*Tev*II-Gen unter direkte Kontrolle des pBAD promoters, wodurch das Enzym exprimiert und freigesetzt wird, sodass die 31bp lange Erkennungssequenz, die nur auf mpDNA sowie nicht-rekombinierter mopDNA vorliegt, geschnitten wird.

Als zweite Strategie zur Verringerung der Menge an Rest-DNA während der BG Herstellung wurde die suizidale Aktivität der *Stapylococcal nuclease A* (SNUC) untersucht. Wegen der geringen Größe der mcDNA und ihrer engen Interaktion mit dem spezifischen, membranverankerten DNA-Bindeprotein (LacI-L‘) wird angenommen, dass mcDNA von der suizidalen Aktivität von SNUC verschont bleibt. SNUC wird erst nach erfolgter Rekombination und Lyse durch die Beigabe von $CaCl_2$ und $MgCl_2$ aktiviert. SNUC wurde zwar bereits früher für die Herstellung von genfreien BGs verwendet, jedoch noch nie in Kombination mit ParA Rekombination und Protein E-vermittelter Lyse von Bakterien.

Ein weiteres Ziel dieser Arbeit war die Entwicklung einer quantitativen Real Time PCR (qPCR) Technik, die ohne weitere Vorbedingungen und Annahmen die Menge von mcDNA und mpDNA quantifizieren kann, um die Menge von *in vivo* beladener mcDNA im Inneren von BGs sowie die Menge von nicht-rekombinierter mopDNA und verbleibender mpDNA festzustellen. Frühere Versuche auf diesem Gebiet waren insofern beschränkt, als dass die quantifizierte Menge aus der Menge der nicht-rekombinierten mopDNA geschlossen werden musste, während diese wiederum als Schätzung aus der densitometrischen Analyse des Rekombinationsendprodukts gewonnen wurde.

BGs können beide Zweige der Immunantwort, also sowohl die angeborene als auch die adaptive Immunität, stimulieren.

Im Prinzip können beide untersuchten Systeme zur *in vivo*-Beladung und Produktion von mcDNA innerhalb des BGs führen und gleichzeitig eher frei von mpDNA und nicht-rekombinierter mopDNA sein, sodass folgende Aufreinigungen unnötig sind. Die Entwicklung einer Quantifizierungsmethode zur Detektion von mcDNA-beladenen BGs in einem Schritt – basierend auf der Real Time PCR-Methodik – dient demnach als Qualitätskriterium und revolutioniert die Quantifizierung von mcDNA/mpDNA.

## Zusammenfassung der Ergebnisse

Die vorliegende Arbeit „Minicircle DNA Immobilization in the Bacterial Ghosts: Investigation for the Reduction of Un-recombined Mother Plasmid DNA and Miniplasmid DNA in BGs" diskutiert die verschiedenen Möglichkeiten zur Verbesserung der aktuellen Methodik zur Herstellung von Minicircle DNA (mcDNA) beladenen Bacterial Ghosts (BGs), die frei von restlicher miniplasmid DNA (mpDNA) sowie nicht-rekombinierter mother plasmid DNA (mopDNA) sind. Spezifische Änderungen im Selbstimmobilisierenden Plasmid (SIP) beziehungsweise die Verwendung der *Staphylococcal Nuclease A* (SNUC) ermöglichten die Produktion von mcDNA-beladenen BGs, die überwiegend frei von mpDNA sowie nicht-rekombinierter mopDNA sind. Die Einführung einer neuen Methode zur Detektion von unterschiedlichen Formen von Plasmid DNA in BGs ermöglicht einen einfacheren, effizienteren und verlässlicheren Quantifizierungsprozess als zuvor. Die Untersuchungen, die im Rahmen dieser Arbeit durchgeführt wurden, lassen sich am besten in den unten folgenden sechs Themen zusammenfassen.

**Das Bacterial Ghost Platform System – Produktion und Anwendungen**

Die Bacterial Ghost Platform-Technologie ist ein innovatives Trägersystem für Vakzine, Medikamente oder aktive Substanzen sowie für technische Anwendungen in der „Weißen Biotechnologie". BGs sind Zellhüllen, die von Gram-negativen Bakterien gewonnen werden. Während das Cytoplasma entleert wird, bleibt die Oberflächenmorphologie von BGs erhalten, was die intrinsischen Adjuvanseigenschaften der BGs erklärt und zu einer erhöhten humoralen und zellulären Immunantwort auf ein präsentiertes Zielantigen führt. BGs werden durch Batch Fermentation und anschließender Produktgewinnung und -aufreinigung durch Tangential Flow Filtration hergestellt. Aus Sicherheitsgründen wird während des BG Produktionsprozesses alle verbleibende DNA inaktiviert, entweder durch die Verwendung von SNUC und/oder die Behandlung mit β–propiolacton. Das breite Spektrum möglicher Anwendungen für BGs in Verbindung mit den vergleichsweise geringen Produktionskosten machen die Bacterial Ghost Platform-Technologie zu einem sicheren und ausgefeilten Produkt zur zielgerichteten Präsentation von Impfstoffen und aktiven Substanzen sowie als Träger für immobilisierte Enzyme für Anwendungen in der „Weißen Biotechnologie".

**Reduzierung von nicht-rekombinierter mutterplasmid DNA (mopDNA) und miniplasmid DNA (mpDNA) in BGs**

Der enzymatische Verdau von mpDNA sowie nicht-rekombinierter mopDNA in BGs durch die Homing Endonuclease I-*Tev*II war das grundlegende Ziel dieses Teils dieser Arbeit. Das I-*Tev*II-Gen wurde kommerziell synthetisiert und sollte in eine weiterentwickelte Version des SIP kloniert werden. Verschiedene bakterielle Stämme mit strenger interner Expressionskontrolle durch $lacI^q$ sowie mehrere verschiedene Expressionsvektorsysteme wurden zur Klonierung dieses potentiell toxischen Genes herangezogen. Auch wenn mehrere alternative Herangehensweisen versucht wurden, konnte die Klonierungsstrategie nicht vollständig erfolgreich umgesetzt werden. Stattdessen wurden ein neues weiterentwickeltes SIP sowie mehrere Intermediate hergestellt. Mehrere Versuche, I-*Tev*II zu klonieren führten zu SIP Varianten mit mutierten I-*Tev*II Sequenzen. Dazu gehört ein spezieller Klon mit einer 48bp Deletion, wobei kein Frameshift hervorgerufen wurde. Diese Deletion führt zu einem kürzeren Protein, dessen volle Funktion (hauptsächlich aus Zeitgründen) nicht getestet wurde. Aufgrund des geringen verfügbaren Wissens über die Funktion des I-*Tev*II-Gens war es schwierig den zugrundeliegenden Mechanismus zu identifizieren, sodass angenommen wird, dass die Kombination aus I-*Tev*II-Gen und einer bislang nicht identifizierten SIP Backbone Sequenz letal für die Empfängerbakterien ist, sodass die angewandten Strategien erfolglos bleiben mussten. Allerdings konnte das Intermediat SIP p4a kloniert werden, das im Vergleich mit seinen Vorgängern über einige verbesserte Eigenschaften verfügt.

**MinicircleDNA (mcDNA) beladene BGs befreit von nicht-rekombinierter mutterplasmid DNA (mopDNA) und miniplasmid DNA (mpDNA) durch nuclease-aktivität**

Eine neue Methodik basierend auf Nuclease-Inaktivierung zur Reduzierung von mpDNA in *E. coli* Nissle 1917 BGs wird ebenfalls hier präsentiert. Zur Herstellung von mcDNA durch sequenzspezifische parA-Rekombination, die die Sequenz für das Enhanced Yellow Fluorescent Protein (EYFG) unter einem eukaryotischen Promoter trägt, wurde das Plasmid p4aEYFG hergestellt. Durch die enzymatische Aktivität von SNUC wurde mpDNA und nicht-rekombinierter mopDNA hydrolysiert: durch quantitative Real Time PCR (qPCR) wurde gezeigt, dass 2.38% der mcDNA (23-59 Plasmidkopien/BG) von der hydrolytischen Aktivität von SNUC nicht betroffen waren. SNUC hydrolysiert jene DNA-Fragmente, die größer und leichter erreichbar sind (also mpDNA oder mopDNA) bevorzugt, sodass mit dieser Methode die Menge von mpDNA in BGs um 99.48% verringert werden konnte (was 8.27 Plasmidkopien/BG entspricht). Dieser Wert ist sehr viel geringer als der durch normale

ParA-Rekombination und Protein E-vermittelte Lyse erreichte Wert von ~412 Plasmidkopien/BG. mcDNA-beladene Ghosts, die mittels dieser neuen Methodik entwickelt wurden, vermittelten in Makrophagenzellen RAW-264.7 die Expression des mcDNA-verankerten Gens.

**Herstellung von Minicircle DNA (mcDNA)-beladenen, Reportergen exprimierenden BGs und ihre Quantifizierung mittels quantitativer Real Time PCR (qPCR)**

Auf eine weiterentwickelte Version des SIPs wurden drei unterschiedliche Reportergene, Enhanced Yellow Fluorescent Protein (EYFP), Red Fluorescent Protein (RFP) und Mutated Green Fluorescent Protein (VenusA206K) kloniert.

Diese neu klonierten Plasmide wurden in CCL-20.2 Zellen getestet und führten zu höheren Fluoreszenzsignalen verglichen mit den Originalplasmiden. Berechnung mittels der neu entwickelten qPCR zeigte für diese SIPs eine Rekombinationseffizienz von 99.73%. Die für diese neue Methodik speziell entwickelten Primer haben die zusätzliche Fähigkeit, je nach Art der verwendeten mopDNA (also rekombiniert oder nicht-rekombiniert) unterschiedlich lange PCR-Fragmente zu liefern, sodass durch die Analyse der finalen Schmelzkurve die Quelle des quantifizierten PCR-Produkts gefunden werden kann. Durch die Einführung dieser neuen Methodik kann mpDNA und mcDNA ohne weitere Voruntersuchungen oder Abschätzungen quantifiziert werden. Im Übrigen verfügt dieses System über den Vorteil der Fähigkeit zur Berechnung der Rekombinationseffizienz, ohne auf densitometrische Analysen des Rekombinationsprodukts angewiesen zu sein.

**Verbesserung von Selbstimmobilisierenden Plasmiden (SIPs) für die Herstellung von Minicircle DNA (mcDNA)-beladenen BGs**

Um mcDNA für Vakzinierungsstudien zu verwenden, müssen alle unnötigen Sequenzen entfernt werden; darüber hinaus ist die Verwendung von Antibiotikaresistenzen, die für gewöhnlich nicht im medizinischen Bereich verwendet werden, vorteilhaft. In diesem Projekt wurde die Struktur des SIP insofern verändert, als dass durch die Positionierung der Resolution Site 1 (res1) die Größe der mcDNA verringert werden konnte. Nunmehr besteht diese nur noch aus der LacOs-Sequenz und der Multiple Cloning Site zur Einführung des Zielgens, wohingegen durch die Invertierung der LacI-L‘/ParA Expressionskassette die Klonierung von Genen für den enzymatischen Verdau von mpDNA nach der Rekombination erleichtert wurde. In dieser neuen Version des SIP wurde die Antibiotikaresistenzkassette von Ampicillin zu Kanamycin ausgetauscht; dies wird von regulatorischen Behörden wie der

Food and Drug Administration (FDA) und der European Medicine Agency (EMA) empfohlen.

**Bacterial Ghosts als Träger von Proteinuntereinheiten und DNA-kodierten Antigenen für Vakzinanwendungen**

Bacterial Ghosts (BGs) stellen Vakzinträgersysteme mit herausragenden natürlichen Adjuvanseigenschaften dar. BGs sind leere Zellhüllen Gram-negativer Bakterien, ohne deren cytoplasmatischen Inhalt, aber mit unveränderter Morphologie und den intakten strukturellen Eigenschaften ihrer lebenden Entsprechung, was ihre einfache Erkennung durch professionelle Antigen präsentierende Zellen mittels Pattern Recognition Receptors ermöglicht. BGs eignen sich für die Anwendung über Schleimhäute mittels oraler, ocularer, intranasaler oder aerogener Route, was den bevorzugten Wegen der Vakzinapplikation entspricht. BGs wurden als Träger aktiver Substanzen und von Fremdantigen (als Protein oder DNA) entwickelt; diese Übersicht betont die hervorragenden Eigenschaften von BGs als Vakzinplattform mit einer Vielzahl an erfüllten Zwecken, für die weitreichende Anwendung in humaner und veterinärer Medizin.

# Publication I

# The Bacterial Ghost Platform System

# Production and applications

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# The bacterial ghost platform system
## Production and applications

Timo Langemann,[1,2] Verena Juliana Koller,[1,2] Abbas Muhammad,[2] Pavol Kudela,[1,3] Ulrike Beate Mayr[1] and Werner Lubitz[1,2,*]

[1]BIRD-C GmbH and CoKEG; Kritzendorf, Austria; [2]Centre of Molecular Biology; University of Vienna; Vienna, Austria; [3]Cancer Research Institute; Slovak Academy of Sciences; Bratislava, Slovakia

**Key words:** E-mediated lysis, bacterial ghosts, vaccines, drug carrier, fermentation, bacterial inactivation, tumor treatment, white biotechnology

**Abbreviations:** BG, bacterial ghost; IM, inner membrane; OM, outer membrane; PPS, periplasmic space; cfu, colony forming units; $dO_2$, dissolved oxygen; TFF, tangential flow filtration; BPL, β-propiolactone; $dH_2O$, de-ionized water; $OD_{600}$, optical density at 600 nm; FSC, forward scatter; FL1, fluorescence signal 1; IPTG, isopropyl β-D-thiogalactopyranoside; *App*, *A. pleuropneumoniae;* PAMP, pathogen-associated molecular pattern; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; TLR, toll-like receptor; AG, antigen; CPS, cytoplasmic space; DOX, doxorubicin; Caco-2, colorectal adenocarcinoma; DC, dendritic cell; pSIP, self-immobilizing plasmid; GFP, green fluorescent protein; mc, minicircle; APC, antigen-presenting cell; ADH, alcohol dehydrogenase; IL, ionic liquid

The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology. BGs are cell envelopes derived from Gram-negative bacteria. BGs are devoid of all cytoplasmic content but have a preserved cellular morphology including all cell surface structures. Using BGs as delivery vehicles for subunit or DNA-vaccines the particle structure and surface properties of BGs are targeting the carrier itself to primary antigen-presenting cells. Furthermore, BGs exhibit intrinsic adjuvant properties and trigger an enhanced humoral and cellular immune response to the target antigen. Multiple antigens of the native BG envelope and recombinant protein or DNA antigens can be combined in a single type of BG. Antigens can be presented on the inner or outer membrane of the BG as well as in the periplasm that is sealed during BG formation. Drugs or supplements can also be loaded to the internal lumen or periplasmic space of the carrier. BGs are produced by batch fermentation with subsequent product recovery and purification via tangential flow filtration. For safety reasons all residual bacterial DNA is inactivated during the BG production process by the use of staphylococcal nuclease A and/or the treatment with β-propiolactone. After purification BGs can be stored long-term at ambient room temperature as lyophilized product. The production cycle from the inoculation of the pre-culture to the purified BG concentrate ready for lyophilization does not take longer than a day and thus meets modern criteria of rapid vaccine production rather than keeping large stocks of vaccines. The broad spectrum of possible applications in combination with the comparably low production costs make the BG platform technology a safe and sophisticated product for the targeted delivery of vaccines and active agents as well as carrier of immobilized enzymes for applications in white biotechnology.

*Correspondence to: Werner Lubitz; Email: werner.lubitz@univie.ac.at
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## Introduction

Bacterial ghosts (BGs) are envelopes from Gram-negative bacteria which have been produced by controlled expression of the cloned lysis gene *E.* The essential role of gene *E* in the lysis of *Escherichia coli* after infection with bacteriophage φX174 was discovered in 1966.[1] More than 16 years later, when genetic engineering had been developed, it could be shown that its sole expression after cloning is sufficient to cause subsequent lysis of *E. coli*.[2,3] *E* was the first lethal gene for bacteria which could be silenced on plasmids. When established in non-host range bacteria of the phage expression of *E* converts Gram-negative bacteria into BGs whereas Gram-positive bacteria are killed without lysis.

Gene *E* codes for a 91-aa polypeptide,[4,5] which, in contrast to lytic proteins from other phages, has no inherent enzymatic function.[6,7] E represents a membrane protein with the ability to oligomerize into a transmembrane tunnel structure.[8,9] Analysis of the primary structure of protein E revealed a hydrophobic region at its N-terminal end suggesting a cotranslational integration into the cytoplasmic membrane of *E. coli*.[10] The observations that stationary phase host cells do not respond to E-lysis induction but lyse upon provision of fresh medium and other findings such as the inhibitory effect of non-physiological pH-values on the E-lysis process, indicate that E-mediated lysis is dependent on the growth phase of the host cells and its autolytic system.[9,11-13]

Analysis of the hydropathicity regions of protein E indicated an E-specific lysis tunnel spanning the inner (IM) and outer membrane (OM) which most probably is located at membrane adhesion sites within the host cell.[14] E-mediated lysis forms the BG by releasing all cytoplasmic content to the environment while periplasmic components remained associated with the empty cell envelope.[9] The collapse of the bacterial membrane potential precedes the onset of E-lysis.[15] When the E-lysed *E. coli* were viewed by high-magnification scanning and transmission electron microscopy, the E-specific lysis tunnel was observed (**Fig. 1A**).

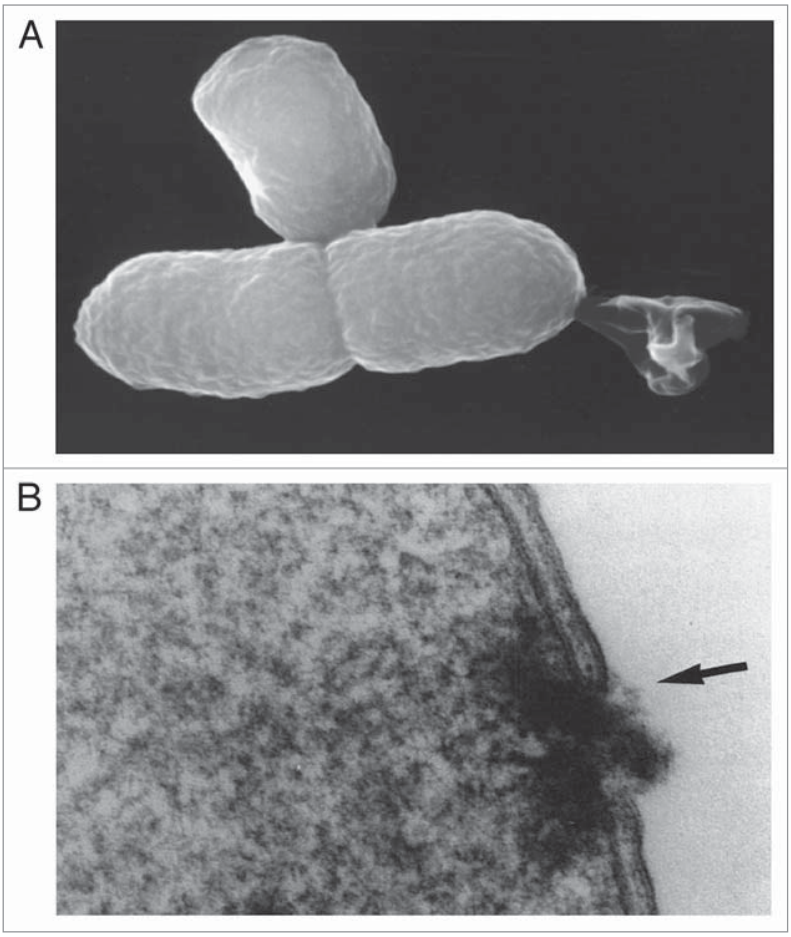


**Figure 1.** (A) Lysis tunnel formation and expulsion of the cytoplasmic contents—reproduced from Ebensen et al.[32] (B) Lysis tunnel formation, accompanied by the fusion of IM and OM (arrow)—reproduced from Witte et al.[16]

In addition, electron microcopic images showed that E- lysis of *E. coli* was accompanied by a fusion of the inner and outer membrane (**Fig. 1B**) sealing the periplasmic space (PPS).[16] Several investigations of E-lysed *E. coli* cells revealed that the E-specific lysis tunnel is located either at the centre or the poles of the bacteria, both potential division zones.[17] Since protein E-mediated lysis is dependent on the physiological state of the host bacterium[18,19] and analysis of E-lysis in different *E. coli* cell division mutants suggest that mechanisms involved in cell division are mandatory for E-lysis.[17,20]

The observed lysis tunnel diameter varies between 40 to 200 nm and does not show any regular structure. The driving force for the rapid discharge of the cell content is the osmotic pressure difference between the cytoplasm and the surrounding medium. However, the native structure of the peptidoglycan within the envelope complex remains intact and rigid.[21] The observed stimulation of peptidoglycan turnover by about 10%[21] would agree with genetic evidence that protein E inhibits MryA translocase A.[22]

Based on these findings and extended experiments using an E-streptavidin fusion protein (E-FXa-StrpA), Schön et al.[23] described the process of E-mediated tunnel formation with a three-phase model: (1) integration of protein E into the IM with the C-terminus facing the cytoplasm; (2) conformational change of protein E translocating the C-terminal domain to the PPS accompanied by oligomerization and targeting of the division initiation complex via lateral diffusion; (3) fusion of IM and OM at membrane adhesion sites induced by exposition of the C-terminus of protein E to the cell surface. This model implies that the lysis tunnel is note solely bordered by protein E oligomers but its formation requires protein E-triggered fusion of the inner and outer membrane.[23] A schematic drawing of this model is shown in **Figure 2B**.

Upon discovery of the remarkable features of protein E-mediated lysis in *E. coli*, the principle of E-lysis could be shown with other Gram-negative bacteria[24,25] but not for Gram-positive bacteria.[26] So far, BGs of numerous Gram-negative strains (different *E. coli* strains, *Salmonella typhimurium, Salmonella enteritidis, Klebsiella pneumoniae, Bordetella bronchiseptica, Heliobacter pylori, Vibrio cholerae, Actinobacillus pleuropneumoniae, Haemophilus influenzae, Mannheimia haemolytica, Pasteurella multocida, Pseudomonas aeruginosa, Pseudomonas putida, Ralstonia eutropha, Pectobacterium cypripedii* and others) have been generated successfully. This suggests that the BG platform might be extended to any Gram-negative bacterium.[25] The idea of utilizing BGs derived from different Gram-negative bacteria as candidate vaccines emerged due to the demand for both potent and safe new vaccines.[27-31] The BG system offers many advantages over traditional vaccination techniques including targeting and the intrinsic adjuvant properties of the BG particles. In addition, recombinant DNA technology facilitates the development of multivalent protein or DNA vaccines. Another great feature of BGs is the fact that no denaturing effects occur during E-lysis and hence all antigenic determinants are preserved throughout BG generation. The use of BGs as candidate vaccines and advanced drug carriers can be found in several recent reviews.[24,27,29,32-36]

## BG—Production Process

Initial cloning and expression studies with gene *E* used the inducible *lac* promoter/operator system with an overexpression of the *lacI* repressor gene (*lac* PO-*lacI*$^{q1}$).[2,3] Later the temperature-sensitive λ-system ($\lambda p_L/\lambda p_R$-*c*I857) has proven to be more suitable for quick and efficient lysis without the need of any addition of chemical inducers.[18] Since the λ repressor *c*I857 shows incipient expression of downstream gene *E* at temperatures above 30°C the temperature sensitivity of the system was optimized to meet more favorable fermentation temperatures of 35°C or higher. Mutations in the $O_R2$ operator region of the $\lambda p_R$ promoter resulted in tight repression of downstream genes up to 36°C and 39°C, respectively.[37,38] These temperature-inducible *E* expression cassettes are

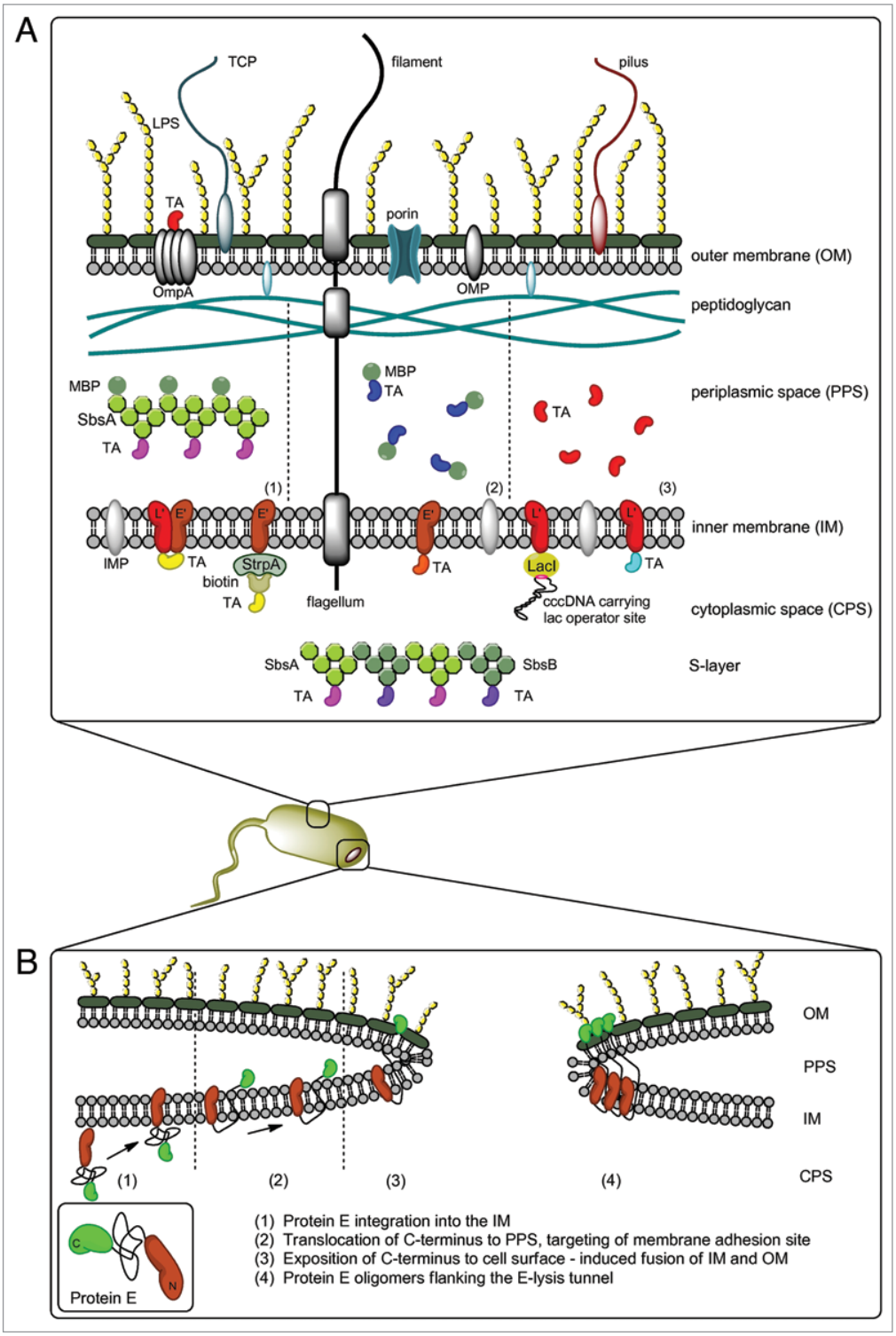


**Figure 2.** (A) Different methods for AG presentation in the BG envelope complex—BG themselves carry native AG (LPS, OMP, IMP, TCP, flagella, pili)—TA may be presented on the cell surface via fusion with OmpA—the PPS can be loaded with TA via MBP-SbsA-fusion proteins (1), by fusion of the TA with MBP (2) or as sole TA using the gene III signal sequence (3) Protein TA may be incorporated into the IM via E', L' or E'/L'-anchoring, biotinylated AG can be attached to E'-FXa-StrpA membrane anchors, DNA carrying the lac operator site can be attached to L'-anchored lacI repressor molecules—TA fused with SbsA-/SbsB proteins form S-layers in the PPS. (B) Model of lysis tunnel formation according to Schön et al.[23]

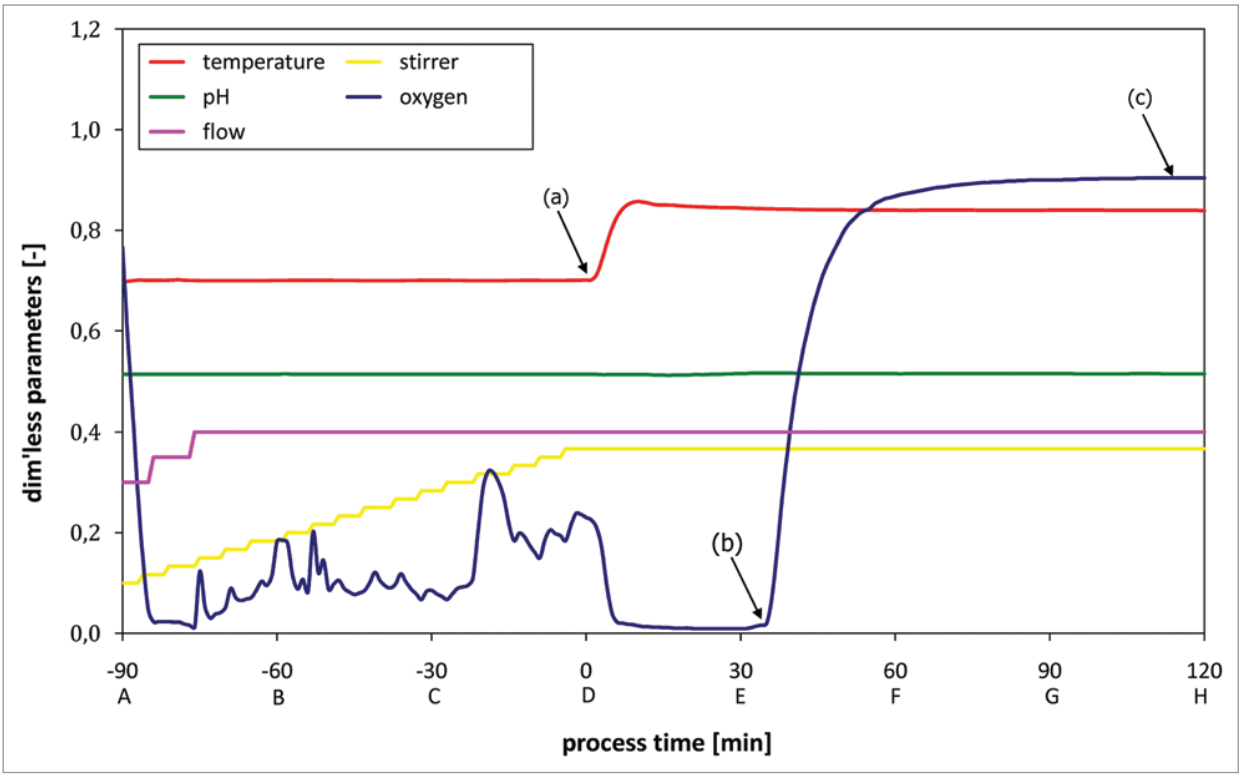


**Figure 3.** Fermentation protocol (growth/lysis phase) monitoring all relevant process parameters; (a) lysis induction, (b) lysis onset as indicated by $dO_2$ up-shift, (c) stationary $dO_2$ plateau indicating end of lysis phase.

widely used in current BG production processes since they are robust enough to allow fermentation of the bacterial culture at 35°C and induction of protein E-mediated lysis at 42 or 44°C.

In standard fermentations of various bacteria the quality criterion for a successful E-lysis process is a BG formation of at least 99.9% of the bacterial culture within a time window of 2 h. Depending on the host organism E-lysis efficiencies of more than 99.99% and higher can be achieved in this time frame. In **Figure 3** the time-point of E-lysis induction is defined as time-point zero (0 min) with the preceding growth phase denoted in negative minutes.

BG production has been established in fermentation volumes up to 20 l using Labfors-3 and Techfors-S fermenters (Infors HT, Bottmingen, CH). Starting with a pre-culture that is growing exponentially, the production fermenter is inoculated with the starter culture at a volume ratio of 1:10. The standard fermentation process can be divided into three major stages: growth phase (90 min), E-lysis phase (120 min) and downstream processing.

The overall timeline for the production process is designed in such a way as the time from the automatic inoculation of the starter culture to the final concentration of the product the whole process takes 18 h and can be performed in one working cycle. The key events of the BG production process (**Fig. 4**) are discussed in more detail below.

**Growth phase.** The growth phase in an example fermentation with *E. coli* harboring plasmids for temperature-inducible E-lysis is conducted at 35°C, pH 7.20 and aeration parameters sufficient for exponential growth. To maintain a level of dissolved oxygen ($dO_2$) of approximately 20% saturation both stirring and aeration rate are adjusted gradually over the course of the growth phase. After 90 min E-lysis of *E. coli* is induced at cell densities of approximately 1–2 x $10^9$ cells/ml.

When recombinant proteins are expressed to become incorporated into the envelope complex (before E-mediated lysis) expression of the corresponding genes is induced chemically 30 min after inoculation (e.g., lac-, arabinose induction system). If synthesis of the foreign proteins slows down the growth rate so that lower cell densities are reached, this growth phase may be prolonged up to 120 min to compensate and increase the BG yield.

**E-lysis phase.** E-lysis of the culture is induced by temperature up-shift from 35 to 42°C (**Fig. 3a**). Currently, it takes roughly 10 min to reach the new temperature in the fermenter. During this time, stirring and aeration control is locked to prevent foaming during BG formation. The $dO_2$-level subsequently drops below 5% and so remains for about 30 min. In the fermentation log visual evidence for E-lysis onset is a sudden signature increase of $dO_2$ (**Fig. 3b**). The E-lysis phase continues for a total of 120 min with its end being characterized by the $dO_2$ reaching a stationary value of >95% saturation (**Fig. 3c**).

**Downstream processing.** The BG product is harvested from the fermenter via tangential flow filtration (TFF) in a 0.2 µm hollow fiber module at a temperature of 15°C. Firstly, the fermentation broth (20 l) is concentrated to 2.0 l (**Fig. 5a**) and transferred

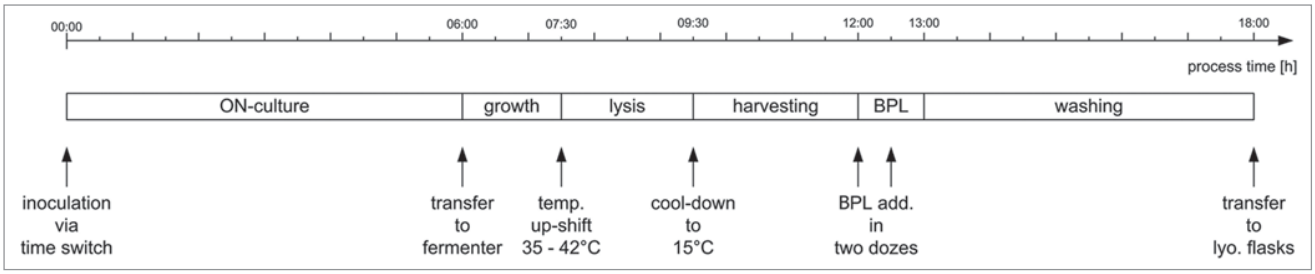


**Figure 4.** Process timeline for the production of BG including the pre-culture (ON) and downstream processing.

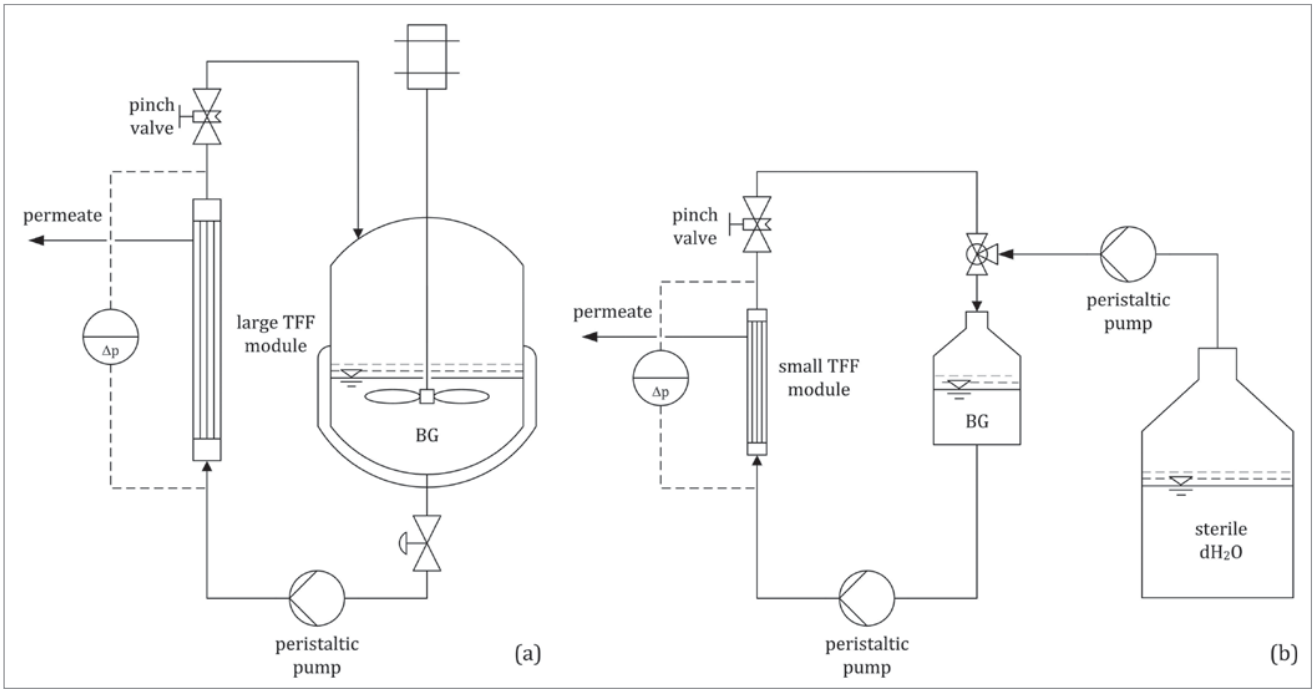


**Figure 5.** (a) Harvesting of the BG product via TFF; concentration from 20 to 2 l in the fermenter. (b) Washing of the BG product with 5.0 l $dH_2O$ via diafiltration; concentration from 2.0 l to 400 ml in a stirred reservoir.

to a stirred reservoir. Then the concentrate is inactivated with β-propiolactone (BPL). Secondly, the BPL-treated broth is washed with sterile, de-ionized water ($dH_2O$) by diafiltration in a smaller 0.2 μm hollow fiber module. A total of 5.0 l $dH_2O$ displaces the remaining medium and all residual cytoplasmic content. During the non-steady-state diafiltration, the product suspension is further concentrated to 400 ml (**Fig. 5a**). The overall concentration factor is 50-fold while virtually all medium (>99%) is withdrawn. The final BG concentrate is divided into aliquots and lyophilized. As freeze-dried product, BGs are stable at room temperature for many years.

The TFF procedure as described above is an alternative to harvesting and washing BG via centrifugation. In contrast to the filtration process, this centrifugation is more laborious, time-consuming and might lead to BG aggregation because of difficulties with a proper re-suspension of the BG pellet. Another advantage of the implementation of TFF for harvesting and washing of BGs keeps all processes in a closed system and reduces the risk of cross-contamination during the handling procedure.

**Process and quality control.** During fermentation all relevant process parameters (T, pH, $dO_2$, aeration, stirring) are monitored and controlled. Starting from the time-point of inoculation (**Fig. 3A,** -90 min) samples are taken every 30 min over the course of the fermentation (**Fig. 3B–H**) and analyzed for optical density ($OD_{600}$) and colony forming units (cfu). All samples are also examined by light-microscopy and flow cytometry. Optionally, the biomass is also investigated for DNA content by real-time PCR and the level of protein E expression.

In standard fermentations with *E. coli,* the onset of E-lysis is linked to a sudden drop in $OD_{600}$ of the culture broth and this simple determination is an important indicator of successful E-lysis induction. BG formation can also be observed as the appearance of translucent bacterial bodies in light microscopy. Both methods are good indicators for the quality of E-mediated lysis of *E. coli* but contain no quantitative information. The

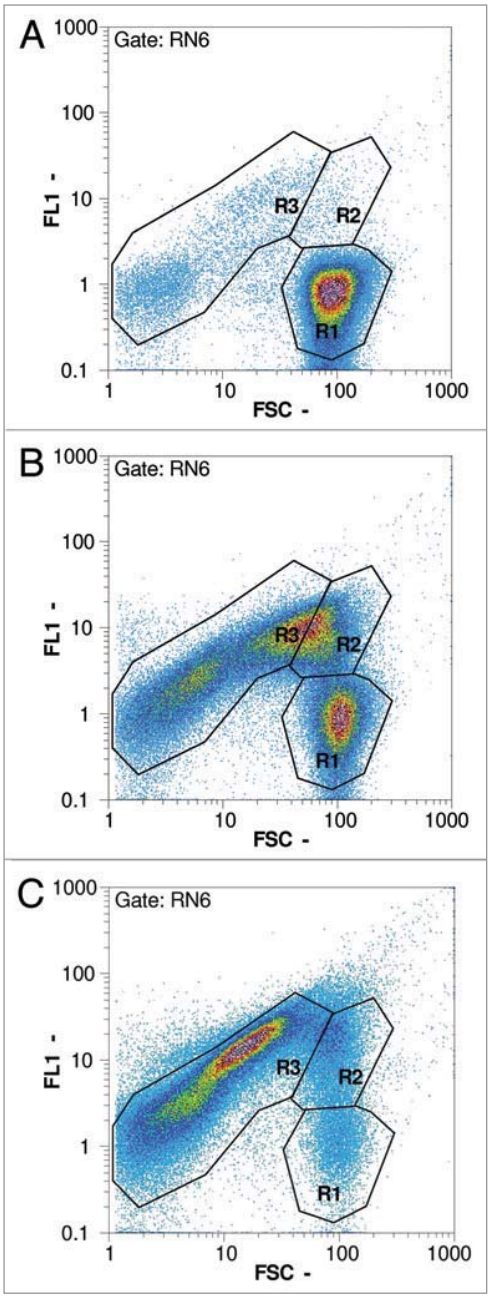


**Figure 6.** Flow cytometry pictures following the progress of lysis in an *E. coli* NM522 culture (pGLysivb); R1: living cells, R2: dead but intact cells, R3: lysed cells (BG); RN6: exclusion of non-cellular background with RH414 (not shown); FSC - forward scatter, FL1 - fluorescence intensity by $DiBAC_4(3)$; (a) sample D (0 minutes, lysis induction), (b) sample E (30 minutes), (c) sample H (120 minutes, end of lysis phase).

actual E-lysis efficiency is determined by cfu counting one day after sample collection. Flow cytometry has been established as a reliable real-time tool for the assessment of E-lysis onset and the progress of BG formation.[39] For flow cytometry diluted samples of the culture broth are stained with two fluorescent dyes and run through a CyFlow analyzer (Partec, Münster, Germany). The first dye (RH414) stains phospholipid membranes and its fluorescence signal defines a gate for the exclusion of all non-cellular background. The discrimination of living cells, dead but non-lysed cells and BGs is achieved by a combination of the forward scatter signal (FSC) and the fluorescence signal (FL1) of the second dye [$DiBAC_4(3)$] which stains only cells that have lost their membrane potential. DiBAC-negative cells with a high scatter signal represent living cells, DiBAC-positive cells with a similar scatter signal represent the dead cell fraction. DiBAC-positive cells with a diminished scatter signal are identified as BGs. The general procedure for online monitoring E-lysis of *E. coli* by flow cytometry has been developed by Haidinger et al.[39,40] and was adapted recently. The flow cytometry result for a given sample is available in less than 10 min after sampling. Representative dot-plots of an *E. coli* culture growth during the E-lysis process is shown in **Figure 6** for the time-points induction (a), course (b) and end of the E-lysis phase (c).

After lyophilization the dry BG product is investigated with respect to sterility and re-suspensibility. For sterility investigations, 10 mg of BGs are re-suspended in rich medium and aliquoted for both nutrient agar plating and enrichment cultures. All sterility tests are performed in triplets to ensure that the final product does not contain any viable cells. The re-suspensibility is evaluated via flow cytometry with a lyophilized sample after re-suspension in $dH_2O$. Since lyophilized BGs generally are easily rehydrated, the sample should give a similar picture and corresponding particle counts as the original sample.

## BG—Inactivation

For the last 2 years, a new quality criterion stipulated that the harvested BG product should be free of any living cells before lyophilization. Although the efficiency of BG formation reaches three to five orders of magnitude during the time window of E- lysis (**Figs. 3 and 4**), any remaining live cells must be inactivated subsequently. The presence of protein E in the envelope complex of bacteria does not necessarily kill all bacteria by E-lysis. However, protein E in the membrane renders all bacteria more acutely sensitive to killing by lyophilization and in the past no living cell counts could be detected in the lyophilized BG samples. In applications where nucleic acid-free BGs are produced, inactivation can be accomplished by the expression of an additional "kill gene" in the host cells in combination with E-lysis.[41] For this, the staphylococcal nuclease A (SNUC) is used, which reduces the DNA content below the detection limit of real-time PCR. SNUC activity is also responsible for cleaning up residual DNA in BGs and can lead to complete inactivation of the culture as it degrades the host DNA into fragments no longer than 100 base pairs.[41] Activation of the positive effect of SNUC expression, minimizing both cell viability and residual DNA-content in the BG product, is dependent on the addition of $Mg^{2+}$ and $Ca^{2+}$ as well as a shift in pH to 8.0.[41] **Figure 7** shows a *Shigella flexneri 2a* culture harboring plasmid pGLNic for co-expression of temperature-inducible protein E and IPTG-inducible SNUC.

Addition of the alkylating agent β-propiolactone (BPL) after harvesting is effective in fully inactivating all viable cells either in combination with or as an alternative to SNUC. BPL is known

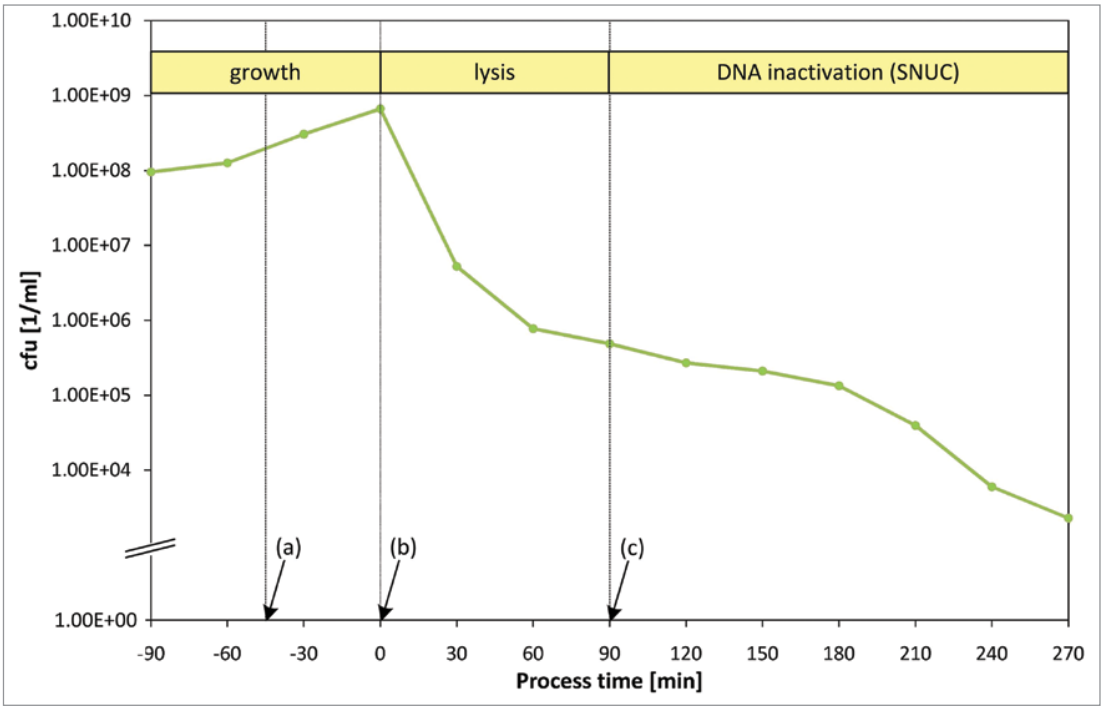


**Figure 7.** Standard fermentation for a *S. flexneri 2a* culture harboring plasmid pGLNic: (a) IPTG-addition at -45 min to induce biosynthesis of SNUC, (b) temperature up-shift to 42°C at 0 min to induce lysis, (c) pH up-shift to 8.0 and addition of $Mg^{2+}$ and $Ca^{2+}$ at +90 min to activate the enzymatic function of SNUC.

to react with nucleic acids, mainly guanine. BPL is widely used for the inactivation of viruses and further to sterilize vaccines, human tissue implants and plasma.[42] The presence of BPL causes alterations (transition mutations, cross-linking, nicks) in nucleic acids. The presence of water fully hydrolyses BPL at room-temperature into non-toxic β-hydroxypropionix acid.[42] In BG production, the amount of BPL needed for complete inactivation of the BG product suspension depends on three parameters: the amount of DNA present in the suspension, time and temperature. Most DNA in the BG suspension is present in the liquid phase due to the expulsion of the cytoplasmic content, which makes it reasonable to apply BPL in the BG concentrate after harvesting but before diafiltration. At this point approximately 97% of the original fermentation liquid—and therefore 97% of the free DNA—has been removed from the product. Two equal dozes of BPL given at 30 min intervals are sufficient for total inactivation of all surviving cells at 42°C within 60 min. The final BG product is washed with another 5.0 l $dH_2O$ by diafiltration before dispensing into aliquots for lyophilization.

## BGs—Applications

**BGs solo.** Immunization against pathogenic Gram-negative bacteria using BGs has been studied in various animal models.[24,36]

BGs have been used in model investigations for human lung pathogens and for the development of veterinary vaccine candidates vaccination of swine with *A. pleuropneumoniae* (*App*). *App*BGs resulted in protection against aerogenic infection with the potentially lethal pathogen. They also prevented colonization of the lungs and tonsils which indicated that immunization with BGs is superior to treatment with bacterins.[43] More importantly, no clinical side-effects have been reported.

The application of BGs via mucosal inoculation is superior to parental inoculation. The mucosal application of *App* BGs as oral immunization[44] or as aerosols[43,45] induced sterile immunity and cross-protection against other serotypes in pigs[45] whereas intramuscular immunization[46] fully prevented the vaccinated pigs against the disease after lethal challenge but did not confer sterile immunity because the challenge bacteria could be re-isolated from the tonsils from the vaccinated pigs.

BG produced from *P. multocida* and *M. haemolytica* (formerly: *P. haemolytica*) were used in rabbit and mice models. The antibodies produced were cross-protective; effective not only against the strain used for immunization but also against other Pasteurella strains.[47] *M. haemolytica* BG immunization of cattle offered protective immunity comparable to commercially available vaccines.[48]

For *V. cholera* pre-clinical studies have been completed. The ilea loop challenge model revealed full protection of rabbits. Interestingly, partial cross-protection between the classical O1 strain and the new upcoming O139 strain was observed.[49] In most models mucosal application has proven to be a favorable route for administration of BG candidate vaccines inducing both humoral and cellular immune response.[29,36]

**BGs as adjuvants.** The BG morphology is not subject to denaturation during the lysis process. Thus all major immune

stimulating elements are preserved. Those elements are referred to as pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides (LPS), monophosphoryl lipid A (MPL), peptidoglycan or flagella. As PAMPs are recognized by toll-like receptors (TLR) they trigger also the innate immune response. Consequently, all bacterial strains from which the BGs are derived induce innate immune reactions (Abtin, Koller, Lubitz, personal communication) as first response. They also carry intrinsic adjuvant properties which makes them extremely versatile to induce specific humoral and cellular immune responses in experimental animals.[29]

**BGs as carriers of foreign protein antigens.** Using recombinant DNA technology, foreign antigens (AGs) can be incorporated into or become associated with the envelope complex of the bacteria before lysis and become elements of the BGs (**Fig. 2A**). AGs may be presented on the cell surface via fusion with outer membrane proteins (e.g., ompA)[50] or on the IM as membrane anchor fusions with N-, C- or N/C-terminal targeting.[30] Fusion with these membrane anchors did not affect proper folding and assembly or diminish the functionality of enzymes supporting the assumption that AGs are in their correct conformation. In addition to directly fusing the target AG to the membrane anchor, a system for subsequent loading of BGs with AGs was developed. In this approach the BGs are equipped with membrane-anchored streptavidin. After lyophilization such streptavidin BGs can be loaded with a desired biotinylated compound.[51]

Another method of incorporating foreign proteins into BGs is the directed export to the PPS via MalE fusion proteins or PPS signal sequences. The PPS is sealed during lysis and the vast majority of all periplasmic components are retained within the envelope complex.[9,16] The membrane-derived oligosaccharides[52] of the PPS provide a protective environment against inactivation during lyophilisation.[34]

Fusions of target antigen DNA sequences with the bacterial surface layer (S-layer) genes *sbsA* or *sbsB* of *Bacillus stearothermophilus*, when expressed heterologous in Gram-negative bacteria, form sheet-like self-assembling superstructures within the cytoplasmic space.[53,54] Since S-layers are made up of several 100,000 subunits, they are not expelled with the cytoplasm during E-lysis. Both S-layer genes accept insertion of foreign sequences coding for large foreign proteins.[54,55] Linking MalE to SbsA the protein subunits can also be exported to the PPS prior to S-layer formation.[56] All different options of AG presentation in BG envelopes are summarized in **Figure 2A** as a schematic drawing.

**BGs as carrier of biologically active substances.** The BG system provides a new promising platform for the delivery of drugs and other biologically active substances. BGs are devoid of any cytoplasmic content so the carrier capacity of the inner cytoplasmic lumen provides an intracellular space of approximately 250 femtoliter per BG. This lumen can be filled with drugs of interest as liquid or absorbed to the lipid compartment (independent of the inner volume) or specifically attached to receptors presented in the BGs.

BGs produced from *M. haemolytica* were used for the in vitro delivery of the moderate hydrophilic cytostatic drug doxorubicin (DOX) to human colorectal adenocarcinoma (Caco-2) cells. Endogenous drug release was confirmed. Enhanced cytotoxic and antiproliferative activities in the Caco-2 cells were observed. DOX-loaded BGs were 2–3 orders of magnitude more effective compared to the substance alone.[57] The water soluble substance calcein was used in another delivery model, whereby the former lysis holes were plugged with bacterial membrane vesicles.[58]

BGs from *P. cypripedii* were used as pesticide delivery systems with the lipophilic fungizide tebuconazole. The investigations demonstrated that this formulation conferred a higher resistance to rainfalls due to adherence of the BGs to the plant. This BG application showed protective and curative effects against agricultural plant pathogens.[59]

It is assumed that organic ring structures bind unspecifically to the membrane compartments of BGs. Recent investigations of loading BGs with polyphenolic compounds, like resveratrol, agree with this assumption. Currently, the modulating ability of such substances bound to BGs to induce the innate immune system, i.e., iNOS, was examined (Koller and Lubitz, personal communication). Furthermore, increased drug cytotoxicity was demonstrated. We predict that this effect was due to stabilization and protection of the UV-labile resveratrol derivates, (Digalloylresveratrol and M8) by adsorption to the BG-interior (Koller and Lubitz, personal communication).

Model investigations for enhanced binding of drugs to a substituted matrix on the inner membrane of BGs have used membrane anchored streptavidin which bound the biotinylated coupling partner to the inside of the cytoplasmic membrane. Biotinylated alkaline phosphatase or biotinylated fluorescence-labelled dextrans displayed successful binding within the inner lumen of BGs.[51]

**BGs as carriers of DNA vaccines.** Conventional viral and bacterial vaccine delivery systems with high transfection efficiencies bear a risk of reversion to their original pathogenic forms. "Safer" non-viral systems such as attenuated bacteria, polycation/DNA complexes, nucleoporation have reduced transfection efficiencies.[60-67] The BG system represents an alternative to current viral and bacterial methods in vaccine development with a new highly efficient gene delivery platform. One of the biggest advantages of the new DNA-carrier system is the safety of BGs. Recent in vitro investigations proved that BGs have no cytotoxic or genotoxic impact on different types of human cells after mutual co-incubation. This observation was independant of the BG species used (Koller and Lubitz, personal communication).

Recently, DNA vaccines were approved for use in veterinary practice.[68] DNA vaccines still require intensive research and improvements before they are considered safe for use in human medicine. One reason for this slow pace in development and licensing approval of DNA vaccines is the requirement of high plasmid dosages and low immunogenicity, most commonly attributed to the absence of efficient delivery system.[69,70] Many experiments have been carried out in order to deliver DNA vaccines using BGs as carriers, and a simple procedure for loading BGs with plasmid DNA has been standardized. Lyophilized BGs are re-suspended in DNA solutions followed by a couple of washing steps to remove unbound plasmid DNA from inside the BGs. The amount of DNA loaded inside the BGs is directly related to

the concentration of DNA solution used. This loading procedure is very efficient and up to 6,000 midsize plasmid copies per BG can be loaded.[71]

One of the main advantages of BGs is that they are non-living. They retain all of the surface morphological, structural and antigenic components of their living counterparts. BGs also have an outstanding loading capacity.[72] The inner space of BGs empty envelope can be loaded with a combination of peptides, drugs or foreign DNA which gives us an opportunity to design new types of polyvalent vaccines.[57,71,73,74] We have shown that BGs loaded with plasmid DNA encoding green fluorescent protein (GFP) are efficiently internalized and phagocytized by both professional antigen presenting cells (APCs) and tumor cells. BGs were able to deliver the heterologous genes to both non-dividing cells (monocyte-derived dendritic cells) and dividing cells (macrophages and melanoma). Study results showed that up to 82% of cells expressing the plasmid encoded reporter gene delivered by BGs. Importantly, no cytotoxic impact was observed on target cells.[32,71,74,75] Intradermal and intramuscular immunization of Balb/c mice with BGs loaded with pCMV encoding beta-galactosidase stimulated more efficient humoral and cellular AG-specific immune responses than naked DNA. Beta-galactosidase-specific immune response was detected after intravenous immunization of mice with autologous dendritic cells (DCs) transfected ex vivo with pCMVbeta-loaded BGs.[32] An increase of IFN-gamma producing AG-specific $CD8^+$ T cells was observed in animals vaccinated with DNA loaded BGs in response to restimulation by APCs pulsed with peptide containing the immunodominant MHC class I epitope. BGs enhanced expression of MHC class I molecules and costimulatory molecules on DCs.[32] Cross-presentation of AGs delivered to DCs by BGs could activate both $CD4^+$ and $CD8^+$ T cells and stimulates the immune system to enhance immune response against AGs expressed by target cells. Bacterial LPS enhances maturation of DCs, affects endosomal acidification of DCs and also improves cross-presentation of AGs.[76,77] Inner and outer membrane structures of BGs including LPS remain intact in BGs and the surface LPS effectively stimulate the AG-cross-presentation by DCs.[17,72]

In general the production and loading of BGs with plasmid DNA are two separate tasks. With the introduction of our new self immobilizing plasmids (pSIP) this multistep procedure was simplified into one step in-vivo, cost effective procedure. During this process the plasmid DNA carrying an operator sequence is bound to a specific DNA binding protein present on the IM of the bacteria.[78] The bacterial backbone sequences and antibiotic resistant genes are considered to be a biological safety risk for DNA vaccination and plasmid DNA used in gene therapy. To overcome this hurdle, new more sophisticated versions of pSIP BG-DNA-vaccines, based on minicircle (mc) DNA devoid of such biologically risky remnants were developed. This improved version of pSIP is based on the ParA resolvase system to produce mcDNA which is bound to the IM receptor. The corresponding sister pair miniplasmid produced during this process is expelled to the culture media during the gene-E mediated lysis.[79] A modified system for minicircle production, digesting the miniplasmid has been reported, based on endonuclease activity of *I*-SceI gene encoded from parent plasmid.[80] A new modified pSIP generation is currently under construction with encoded endonuclease activity to digest non-recombinant mother plasmids and the ParA produced miniplasmids.

The main benefit of DNA vaccines is the induction of both cellular and humoral immune responses. Processing of AG through both endogenous and exogenous pathways followed by AG epitopes present both MHC class I and class II molecules.[81-83] Well designed and applied gene therapy should provide successful delivery of desired AG DNA to the APCs. This is followed by its expression, naturally processing and presentation of AG-derived epitopes. T cells raised against delivered, naturally processed and presented AGs by APCs might be more effective in recognition of the same epitopes presented by cells expressing identical AGs. The expression of a delivered gene should induce strong immune responses or change the behavior of targeted cells.

BGs with their intact envelope structures include peptidoglycan and LPS. These elements are not only "waking up" professional phagocytic APCs but are also providing stimulatory impulses to tumor cells. It is known that e.g., melanoma cells have the capacity to behave as non-professional APCs and can phagocyte both apoptotic and live cells[84-87] and as recently shown respond to challenge by BGs.[73] Despite the high DNA loading capacity of BGs, relatively low concentrations of DNA are sufficient for effective gene delivery and expression by melanoma cells. High transfection efficiencies were obtained after incubation of BGs with melanoma cells. Similar results were seen with monocyte-derived DCs encouraging us to design BGs carrying selected immunogenic and immunodominant AGs. These DNA loaded BGs would be used simultaneously for gene transfer to both professional APCs and to tumor cells to induce or amplify AG-specific immune responses.

**White biotechnology—BGs as micro-bioreactors for enzymatic reactions.** Another possible application for the BG platform is the use of BGs as enzyme carriers. The lack of cytoplasm and of a membrane potential due to E-mediated lysis of the bacteria does not lead to a total loss of enzymatic activities. The enzymatic activities of BG membrane-bound β-galactosidase and chloramphenicol acetyl transferase have been described.[88,89] Membrane associated enzymes like ATPases are still functionally active in BGs. Moreover, even though the cytoplasmic content is expelled during lysis, the inside of the cytoplasmic membrane and its associated products are retained. As the IM and OM are fused at the border of the E-specific lysis tunnel enzymes from the PPS like alkaline phosphatase and β-lactamase are largely retained and active.[9,17] ATPase and β-lactamase sustained relative activities in suspended BGs even after one week storage at 4°C. Enzyme activities were also detectable in lyophilized BG-batches stored long-term at ambient temperatures. The enzyme activities were similar to those of recently produced freeze-dried samples, e.g., ATPase activity no significant differences in enzyme activity were observed after five years of storage (Koller, Lubitz—personal communication). This data confirms that BGs enzymes stay functionally preserved during long storage, which indicates the potential of

BGs as reservoirs for biological functions e.g., as dietary enzyme substitution or for other use.

BGs can act as micro-reactors which follows the idea of Pfründer et al. of producing enantiopure fine chemicals such as e.g., asymmetric synthesis of a 3,5-dicarboxyhydroxylate in biphasic ionic liquid/water systems.[90,91] Potent enzymes [e.g., specialized alcohol dehydrogenases (ADHs)] are anchored to the IM while the internal lumen of the BG becomes the reaction space. Re-suspension of the BGs in an aqueous solution with a suitable reduction equivalent allows for proper function of the desired enzyme. Both product and educts in these kinds of reactions are often poorly water-soluble. For this reason, the use of a non-polar solvents such as a suitable ionic liquid (IL)—is essential. IL are organic salts which are liquid at ambient temperatures; due to their low vapor pressure they are considered as safe ("green solvents"). They feature good in-situ extraction properties for product recovery.[92] BGs loaded with the reduction equivalent solution are dispersed in the ionic liquid. Thus this BG system provides the substrate and receives the product. It was shown that the enzyme activity of β-galactosidase could be vastly increased in an IL environment.[93] Preliminary studies of our lab have demonstrated that β-galactosidase was active when BGs were re-suspended in the IL [Bmim]$PF_6$.

Qualitative determinations showed successful hydrolysis reactions of the substrates which were delivered in the ionic liquid. These findings give a first indication of the feasibility and attainable enzymatic activity of such approaches. When a multi-step enzyme system is introduced a limited series of reactions could be performed within one BG. Therefore the BG system could become a versatile vehicle in white biotechnology.


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Chapter: I

# Investigations reducing un-recombined mother plasmid DNA and miniplasmid DNA in the Bacterial Ghosts


**Abstract**

Minicircle DNA (mcDNA) lacking the bacterial backbone (BB) sequence has been used in achieving high levels of transgene expression due to their reduced size. BB is needed for the replication of plasmid DNA inside the bacteria but it contains some unwanted features which interfere with the expression profile of the delivered antigen. These mcDNA are produced inside the bacteria via parA resolvase gene expression where the mcDNA expression cassette is flanked by two resolution sites (*res*). The ParA mediated recombination of the mother plasmid DNA (mopDNA) results into i) mcDNA carrying the eukaryotic expression cassette and ii) miniplasmid DNA (mpDNA) containing BB and antibiotic resistance cassettes. The in vivo loading of mcDNA in Bacterial Ghosts (BGs) have been demonstrated separately through which the mcDNA is produced via parA recombination and anchored inside the BGs via lacI-L' anchor and its recognition sequence present on mcDNA. There were still some left over's of mpDNA in the prepared BGs. In current study it was planned to clone I-*Tev*II homing endonuclease on the self immobilization plasmid pSIP to reduce the un-recombined mopDNA and mpDNA within the BG preparations. I-TevII belongs to group I sun-Y intron of bacteriophage T4 which cleaves its target sequence 13-15nt downstream to their intron insertion sites. The cloning strategy, although trying several alternative paths were not successful but resulted in new self immobilization plasmid p4a and several other intermediates. It is assumed that I-TevII in combination with unknown pSIP backbone sequences was lethal for recipient bacteria and could therefore not been achieved with the strategy applied.

## 1.1 Introduction

Most desirable feature of plasmid DNA vaccines is to be safe and highly efficient in expressing high levels of transgene products inside the human body. It has been shown previously that production of mcDNA lacking the BB sequence express high levels of transgene in vivo [1]. These mcDNA are produced by site specific recombination of mopDNA while a replicative mpDNA is released as a by-product containing undesirable BB sequence and antibiotic resistance gene [1-7]. The mcDNA carries all the necessary elements for expression of cloned gene inside the eukaryotic cells [1]. Up till recently the mcDNA was produced in a two step process i.e. its production inside the bacterial cell and its subsequent linearization and purification through laborious gradient cesium chloride ultracentrifugation which results in high cost of its production thus limiting its production for clinical trials [8]. Therefore another one step process was developed by Chen et.al. in which the recombined mpDNA and un-recombined mopDNA was digested by the action of I-*Sce*I endonuclease whose recognition site is flanked by *attP* and *attB* sites and is only present on mpDNA/mopDNA, however during this method around 3% of the mopDNA and mpDNA was still detectable [8].

Another approach for production and purification of mcDNA was based on Recombinant Plasmid Separation Technology (RPST) in which the mcDNA carrying lactose operator sequence was produced through parA resolvase recombination followed by its separation through interaction of repressor of lactose operon (lacI) with the *lac* operator sequence (lacOs) [9, 10] via affinity chromatography [11]. However this system is unable to separate the mcDNA from un-recombined mopDNA due to presence of recognition sequence (lacOs) on them that is why an efficient recombination system is key to the successful mcDNA separation via this method. This separation technique was also used by Jechlinger et.al. for immobilizing the mcDNA inside the Bacterial Ghosts (BGs) that are highly immunogenic due to their preserved antigenic structures present on their surface [6]. BGs are the empty cell envelope of Gram-negative bacteria produced by the expression of cloned gene *E* of bacteriophage PhiX174 [12, 13]. After the expression of temperature sensitive lysis gene *E* the inner membrane (IM) and outer membrane (OM) fuses together which results in the formation of transmembrane tunnel structure through which the cytoplasmic contents are expelled into the surrounding medium due to difference in their osmotic pressure [13, 14]. The BGs are used for delivering the proteins or plasmid DNA vaccine due to their intrinsic

adjuvant properties bestowed by their preserved surface antigenic structures [15-26]. It has been shown in several studies that these BGs loaded with plasmid DNA performs better in transfection experiments [21, 27, 28]. The mcDNA loaded BGs [6] are produced by site specific parA recombination of mopDNA and followed by anchoring of mcDNA using the SIP system [29] and finally lysis via PhiX174 gene *E* mediation technique [12]. In this system through densitometric analysis the recombination efficiency was shown close to 100% the one of its kind only achievable through parA mediated recombination. However In these preparations around ~30% of mpDNA is still detectable in BG palette which poses a serious hindrance for the use of mcDNA loaded BGs in human clinical trials.

Homing is a genetic phenomenon which was discovered in group I intron of budding yeast [30-32] responsible for the transfer of mobile intervening sequence to homologous allele lacking the desired sequence [31, 33, 34]. This process is initiated through endonuclease activity encoded by mobile intervening sequence that recognizes and cleaves single or double stranded break in homing endonuclease gene negative (HEG-) strand leading to DNA repair mechanisms which through homologous recombination of intron and or inteins repairs the damaged strand at the RNA and protein levels, respectively [31, 32, 34]. I-*Tev*II gene belongs to group I sun-Y intron of bacteriophage T4 lately named as anaerobic ribonucleotide reductase *nrdD* [31, 35]. It cleaves 13-15nt downstream to their intron insertion site generating 2nt 3'-OH overhangs [36]. These endonuclease depending on their type can tolerate several base substitutions in their recognition sequence [37-39] where as other restriction enzyme cannot afford even a single base change. Homing endonuclease have ability to cleave sequences other than their homing sites making them toxic and difficult to clone [40] previously T7 promoter system was successfully used to clone this toxic gene [41]. Homing endonuclease differs from Type-II restriction enzymes on basis that they recognize longer target sequences ranging from 14-40nt [36, 37, 42-46] where as the type II restriction enzymes are palindromic and recognizes between 4-8nt. sequences[47].

This study is aimed to investigate different methods in reducing the amount of residual DNA in BGs and to develop a technology for in vivo production and loading of BGs with mcDNA that are free of undesired mpDNA or un recombined mopDNA sequences. In this study the homing property of I-*Tev*II would be explored in achieving the final goal of mpDNA or un-recombined mopDNA reduction in the BGs. I-*Tev*II gene was chosen because it recognizes a 31bp sequence [36] which is absent on *E. coli* genome. The strategy involved

is to clone I-*Tev*II gene on advanced version of self immobilizing plasmid pSIPHCNparA [6] with slight modifications necessary to facilitate the insertion of this potentially toxic gene i.e. I-*Tev*II [40] in such orientation that it should remain inert before induction of recombination. After recombination of mopDNA the I-*Tev*II gene should be transferred onto the mpDNA and this should bring I-*Tev*II under direct control of pBAD promoter resulting in its expression and production of homing endonuclease enzyme which recognizes and cleaves its recognition sequence which will be present only on mpDNA and un recombined mopDNA finally the linearised DNA fragment will then be degraded by bacterial exonucleases [48].

### 1.2 Basic concept

The newly constructed plasmid carrying I-*Tev*II gene will be named pSIP-I-*Tev*II. Will then have following property

1. The plasmid upon induction with arabinose should recombine into two smaller parts i.e. i) A replicative mpDNA and ii) mcDNA containing eukaryotic expression cassette and lacOs.

2. After recombination the replicative mpDNA containing the BB and antibiotic resistance cassette should be destroyed by the combined action of homing endonuclease I-*Tev*II which will linearise the plasmid segment by recognizing its recognition sequence present only on mpDNA / un-recombined mopDNA followed by completed degradation through bacterial exonuclease activity. This is based on the idea that if small part with gene of interest is not present any more in between the pBAD promoter and I-*Tev*II gene, a larger mRNA containing LacI-L', ParA, and I-*Tev*II is generated resulting in expression of I-*Tev*II gene where as if no recombination takes place only LacI-L' and ParA are encoded by mRNA thus preventing the expression of I-*Tev*II gene in advance (Fig.1).

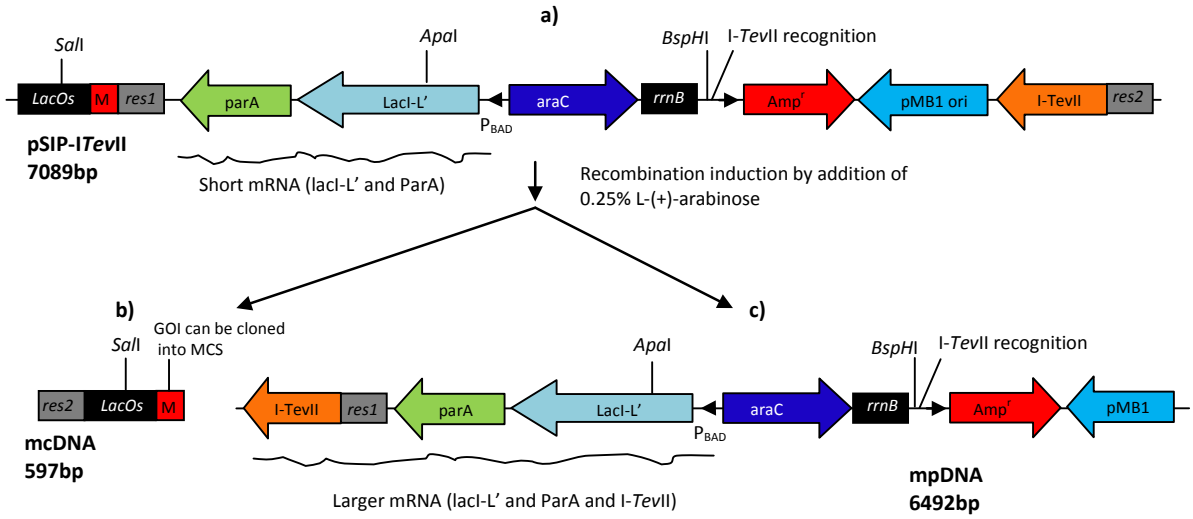


**Fig.1.** ***Basic concept of p-SIP-I-Tev*II;** ***a)*** *The plasmid pSIP-*I-*Tev*II *un-induced form;* ***b)*** *minicircle DNA (mcDNA)produced after induction of pBAD promoter by addition of L-(+)-arabinose carrying only lacOs and res1 along with multiple cloning site;* ***c)*** *miniplasmid DNA (mpDNA) produced after arabinose induction which brings the I-TevII under control of pBAD promoter resulting in production of larger mRNA; lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1;* I-*Tev*II; I-*Tev*II homing endonuclease gene; *res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

## 1.3 Procedure and results

### 1.3.1 Plasmid construction

For constructing plasmid pSIP-I-*Tev*II carrying I-*Tev*II gene which should come under the expressional control of pBAD promoter [49] only after the recombination, it is necessary to carry out some necessary modifications in advanced version of self immobilizing plasmid pSIPHCNparA. For this purpose two different plasmids p4a (carrying inverted expression cassette along with MCS) and p2b (carrying the endonuclease gene) based on pSIPHCNparA has to be constructed separately. The two different regions/parts from above mentioned plasmids will then serve as a template for construction of final version of plasmid pSIP-I-*Tev*II. This plasmid should have the ability to recombine upon arabinose addition into mcDNA and mpDNA thus bringing I-*Tev*II gene under direct control of pBAD promoter

resulting in expression and synthesis of homing endonuclease I-*Tev*II which shall then identify and cleave the I-*Tev*II recognition sequence present on mpDNA or on un-recombined mopDNA. In this way only mcDNA carrying gene of interest is left behind.

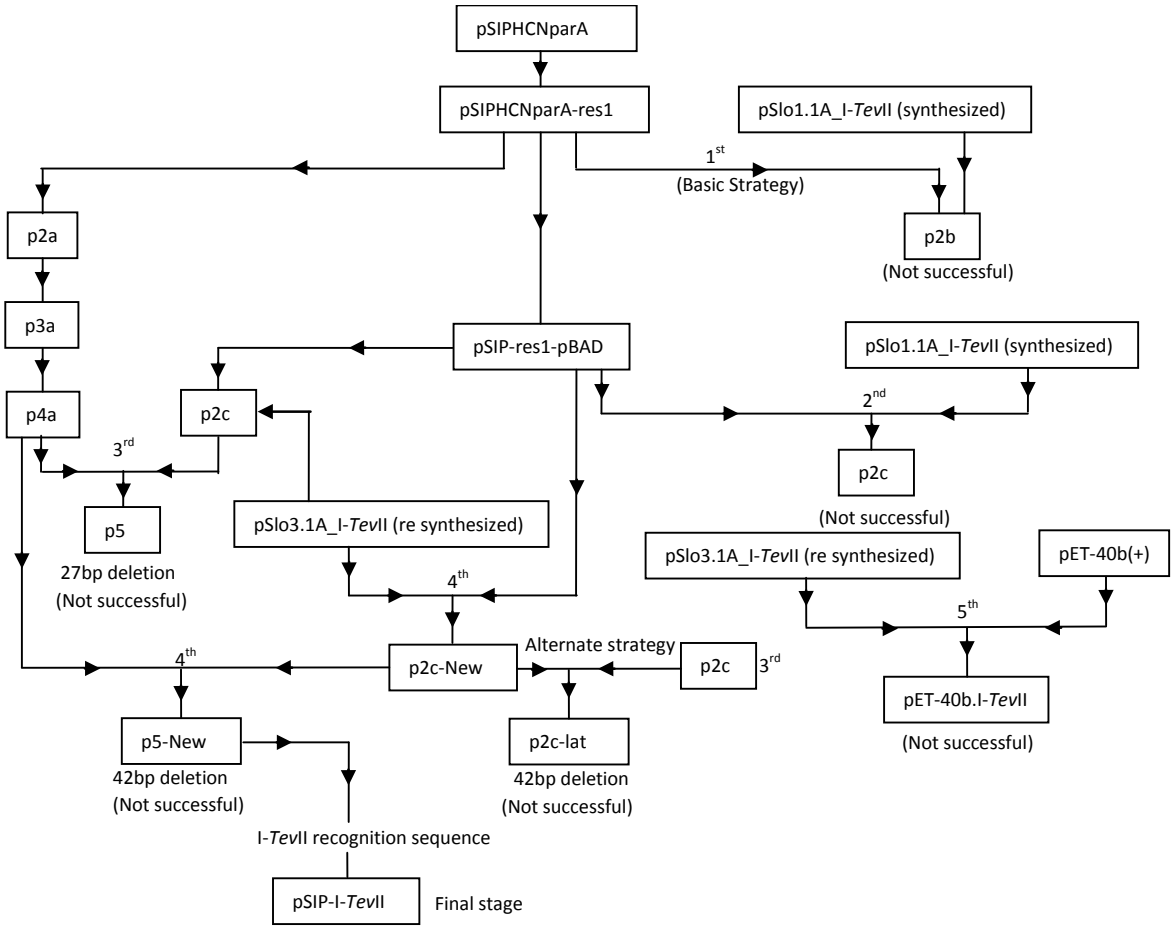


**Fig.1.** ***Flow chart showing different cloning strategies of plasmid pSIP*-I-*TevII***

### 1.3.2 Basic strategy (Construction of plasmid p4a)

To construct Plasmid p3a two intermediate plasmid constructions (pSIPHCNparA-res1, and p2a) was carried out. For easy handling, plasmid pSIPHCNparA [6] (Fig.2a) was digested with *HindIII* (Fermentas) double cut and was re-ligated to remove the resolution site 1 (res1 140bp). The resulting plasmid was named pSIPHCNparA-res1 (6268bp) (Fig.2a)

lacking recombination activity. Suitable restriction sites or multiple cloning sites MCS was introduced into this plasmid pSIPHCNparA-res1 by digesting it with *Nsi*I (Fermentas) and the 60bp MCS (Synthesized) was inserted into this site the resulting annealed plasmid is then called p2a (6128bp) (Fig.2b). This was done by mixing and incubating two primers synthesized for MCS (MCS1 and MCS2) and by introducing this annealed MCS into respective restriction site present on plasmid pSIPHCNparA-res1 using T4 DNA ligase (New England BioLabs, Germany).

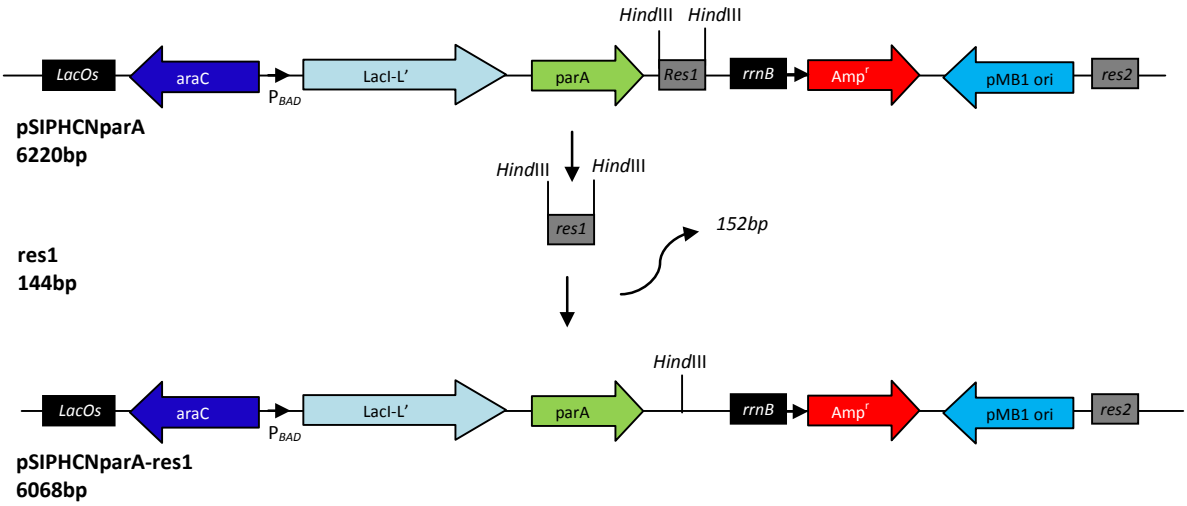


***Fig.2a. Cloning strategy of plasmid pSIPHCNparA-res;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

In order to allow the easy cloning of lethal gene (e.g. *I-Tev*II homing endonuclease that is needed for another study involving plasmid p3a) the expression cassette (LacI-L'/parA) should be inverted for tight repression and prevention of premature expression of cloned gene. *i.e.* before recombination. For this purpose plasmid p2a was digested with *Hind*III (Fermentas) which cuts the plasmid at two different locations and the plasmid was re-ligated using T4 DNA ligase (New England BioLabs, Germany) to get plasmid p3a (6128bp) (Fig.2c).

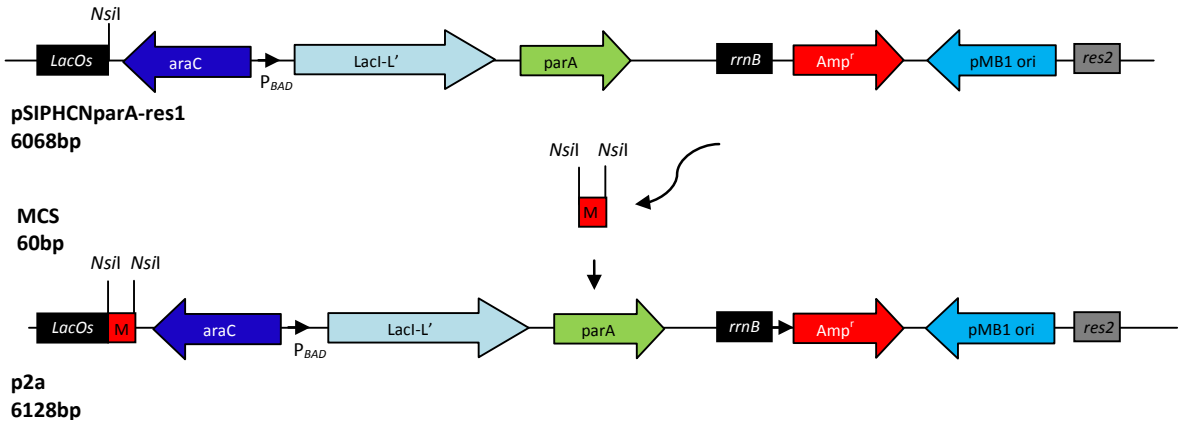


**Fig. 2b*: Cloning strategy of plasmid p2a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*MCS1: 5'-ATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTACCTGCAGTTATAAGCTTATGCAT-3'*
*MCS2: 5'-TACGTAATTAATTGATCAGTCGAGTGCACGCCGGCGGGCCCATGGACGTCAATATTCGAATACGTA-3'*

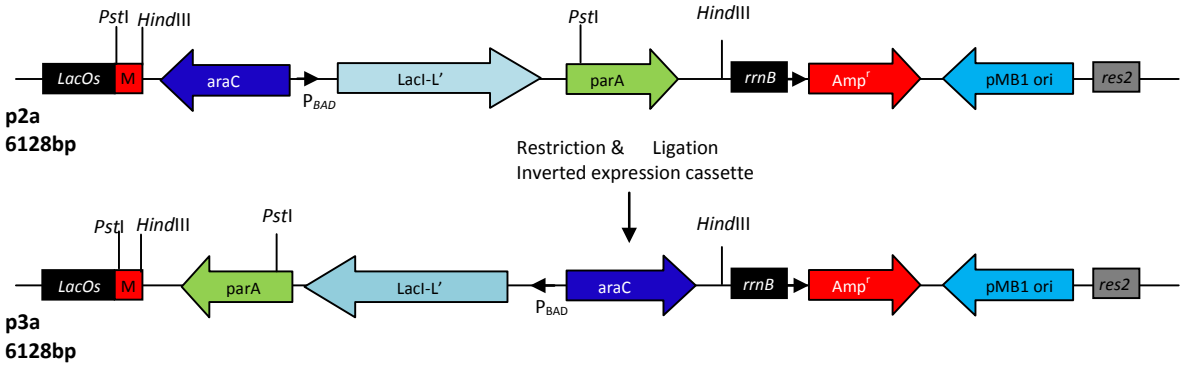


**Fig.2c*: Cloning strategy of plasmid p3a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

The required plasmid was constructed the 1% agarose gel picture shows the correct plasmid p3a (Fig.3).

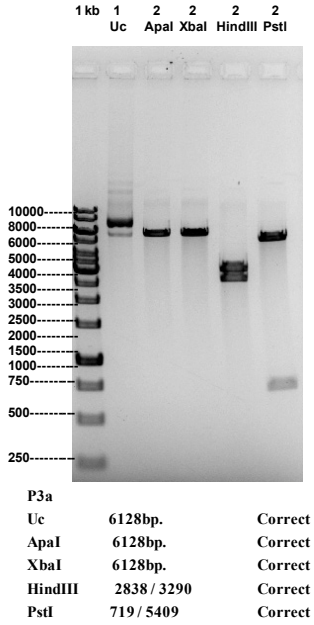


**Fig.3.** ***Restriction analysis;*** *1% agarose gel showing the restriction digest of plasmid p3a with different set of enzymes (positive clone) All enzymes (Fermentas); 1kb, GeneRuler™ 1kb DNA ladder (Fermentas)*

To get the final working plasmid p4a (which has ability to recombine) the resolution site1 was introduced into the plasmid p3a. For this purpose the 140bp res1 site was amplified through PCR using plasmid pSIPHCNparA (Fig.2a) as a template and primers 5'res1K (5'–CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3') and 3res1P (5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3) (synthesized by Microsynth AG, Switzerland) with *Kpn*I and *Psi*I restriction sites (underlined sequences in primer). The PCR reaction with final volume of 25µl was carried out using 0.25µl of (50 pmol/µl) primers each, 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) DreamTaq polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U/ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95$^{o}$C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95$^{o}$C for 30sec, 60$^{o}$C for 30sec and 72$^{o}$C for 1min and final elongation of 72$^{o}$C for 10min.

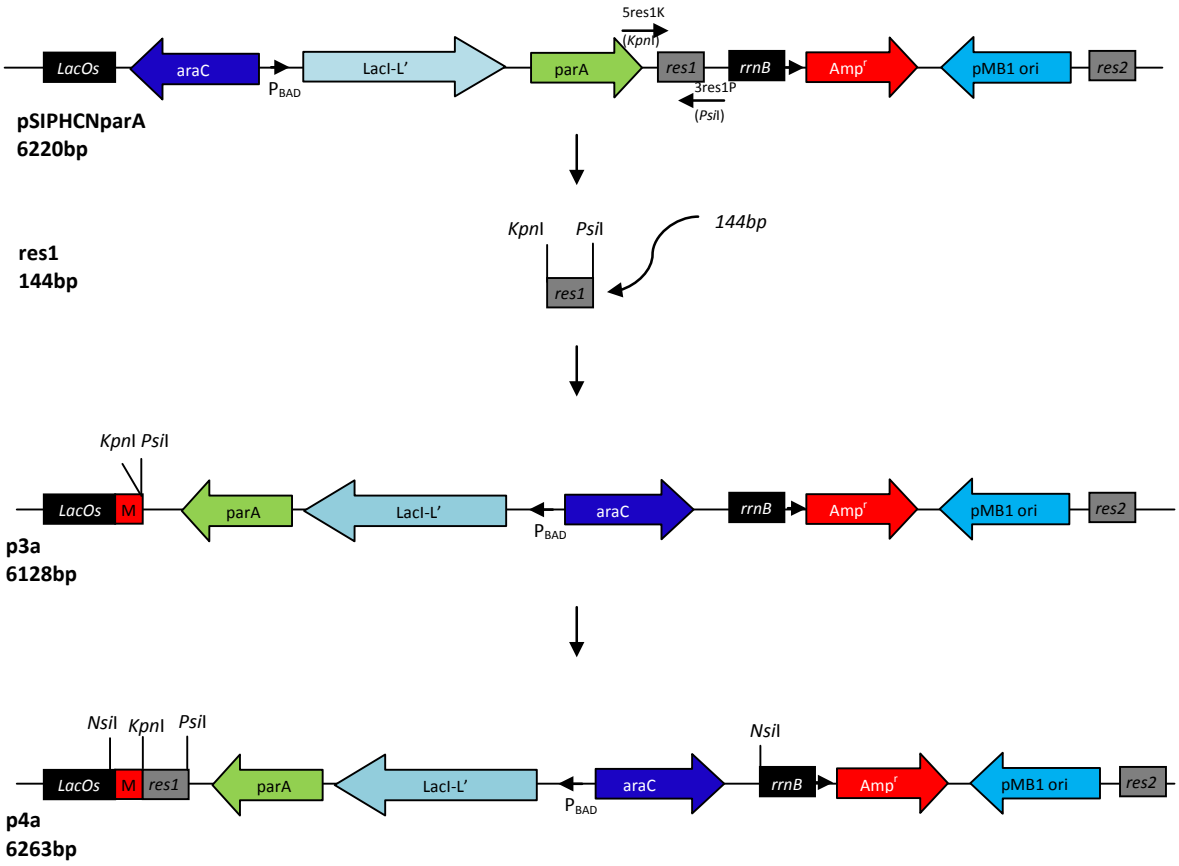


**Fig.4.** ***Cloning strategy of plasmid p4a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*5'res1K 5'– CAG CAG GGT ACC CCT TGG TCA ATT GGG GTA TAC C –3'*
*3res1P 5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3*

The PCR product was analyzed on 2% agarose gel to confirm the amplification. The PCR product was digested with *Kpn*I and *Psi*I (Fermentas) and subsequently cloned into the corresponding sites in plasmid p3a to get the vector p4a (6263bp) (Fig.4). A positive clone of plasmid p4a digested with different sets of restriction enzymes is shown in (Fig.5). The plasmid p4a is under control of pBAD promoter and has two recombination sites. The recombination ability of newly constructed plasmid p4a was checked after transforming the right clone into *E. coli* C2988J and inducing the pBAD promoter via L-(+)-arabinose. The recombination product was subjected to 1% agarose gel to visualize the mcDNA and mpDNA (Fig.6).

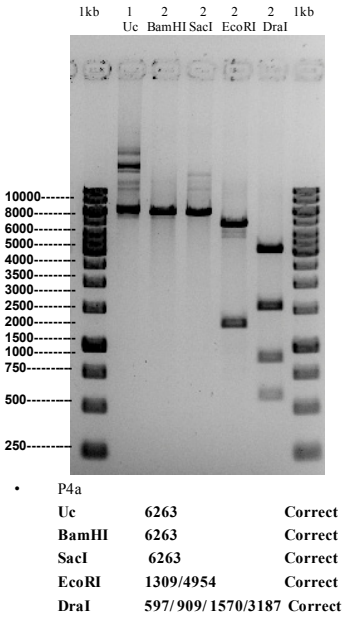


**Fig.5.** ***Restriction analysis;*** *1% agarose gel showing the restriction digest of plasmid p4a with different set of enzymes (positive clone) All enzymes (Fermentas); 1kb, GeneRuler™ 1kb DNA ladder (Fermentas)*

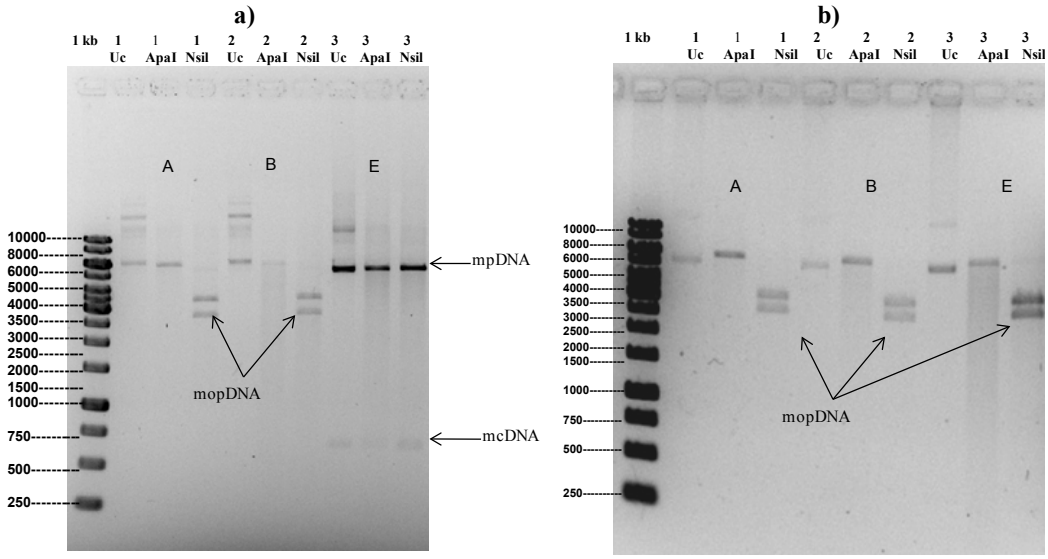


**Fig.6.** ***Recombination analysis of plasmid p4a;*** *1% agarose gel picture showing;* ***a)*** *recombination pattern of plasmid p4a. The cells recombine with the addition of 0.25% L-(+)-arabinose. (3) (digested with different enzymes), no recombination seen prior to arabinose induction (1 and 2) digested with different enzymes;* ***b)*** *No recombination seen in plasmid p3a before (1 and 2) and after (3) addition of 0.25% L-(+)-arabinose due to absence of one resolution site. Uc, uncut; Apa*I*, restriction enzyme; Nsi*I*, restriction enzyme; and 1kb, GeneRuler™ 1kb DNA ladder (Fermentas):mcDNA, minicircle DNA; mpDNA, miniplasmid DNA; mopDNA, mother plasmid DNA*

### 1.3.3 Construction of plasmid p2b

For construction of plasmid p2b (6851bp) the phage T4 I-*Tev*II gene [50] was synthesized by Sloning Biotechnology gene synthesis facility. In order to maximize the gene expression of I-*Tev*II in *E-coli* K12 host the I-*Tev*II gene was codon optimized before its synthesis. Codon optimization involves the specific alteration in sequence of cloned antigen based on free tRNAs levels available in host cells. In this way the codon optimized sequences will use the tDNAs resulting in higher expression of proteins [51-53]. A comparison of original gene sequence and codon optimized sequence for *E. coli* K12 strain is shown in (Fig.7).

Codon Optimized Sequence of I-*Tev*II gene

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Org.: ATG AAA TGG AAA TTA AGA AAA AGC TTA AAA ATT GCC AAT TCT GTA
Opt.: ATG AAA TGG AAA CTG CGC AAA TCC CTG AAG ATC GCG AAC TCA GTT
AA:    M   K   W   K   L   R   K   S   L   K   I   A   N   S   V

Org.: GCA TTT ACC TAT ATG GTA AGA TTT CCT GAT AAG TCT TTT TAT ATA
Opt.: GCG TTC ACC TAT ATG GTA CGG TTC CCG GAC AAG TCT TTC TAC ATT
AA:    A   F   T   Y   M   V   R   F   P   D   K   S   F   Y   I

Org.: GGT TTT AAA AAA TTC AAA ACT ATA TAT GGT AAA GAT ACA AAT TGG
Opt.: GGC TTT AAG AAA TTC AAA ACC ATT TAC GGT AAA GAT ACA AAC TGG
AA:    G   F   K   K   F   K   T   I   Y   G   K   D   T   N   W

Org.: AAA GAA TAC AAT TCG TCA TCT AAG CTT GTT AAA GAA AAG CTT AAA
Opt.: AAA GAG TAC AAT AGC TCT AGC AAA CTC GTA AAA GAG AAA TTA AAG
AA:    K   E   Y   N   S   S   S   K   L   V   K   E   K   L   K

Org.: GAT TAT AAA GCT AAG TGG ATA ATT CTT CAA GTT TTT GAT TCT TAT
Opt.: GAT TAC AAA GCA AAG TGG ATC ATT CTG CAG GTG TTC GAC TCC TAT
AA:    D   Y   K   A   K   W   I   I   L   Q   V   F   D   S   Y
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**Fig.7.** ***Comparison of the original I-TevII sequence vs. Codon Optimized sequence (continued…..)***

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Org.: GAA TCG GCC CTT AAA CAT GAA GAA ATG CTT ATT AGG AAA TAT TTT
Opt.: GAA TCG GCG TTG AAA CAC GAA GAA ATG CTG ATT CGC AAA TAT TTC
AA:    E   S   A   L   K   H   E   E   M   L   I   R   K   Y   F

Org.: AAT AAC GAA TTT ATT CTT AAT AAA TCT ATA GGT GGA TAT AAA TTT
Opt.: AAT AAT GAA TTT ATC CTC AAT AAA TCC ATC GGC GGT TAT AAG TTC
AA:    N   N   E   F   I   L   N   K   S   I   G   G   Y   K   F

Org.: AAC AAA TAT CCG GAT TCA GAA GAA CAT AAG CAA AAA CTT AGT AAT
Opt.: AAC AAG TAT CCG GAT TCC GAG GAA CAT AAA CAG AAA TTG TCG AAC
AA:    N   K   Y   P   D   S   E   E   H   K   Q   K   L   S   N

Org.: GCC CAT AAA GGT AAA ATC TTA TCT TTA AAA CAT AAA GAT AAG ATA
Opt.: GCC CAT AAG GGC AAA ATT CTG AGC CTC AAA CAC AAA GAT AAG ATT
AA:    A   H   K   G   K   I   L   S   L   K   H   K   D   K   I

Org.: CGA GAG AAA TTG ATT GAG CAT TAT AAA AAT AAT AGT AGA AGT GAA
Opt.: CGT GAA AAG CTG ATT GAA CAC TAT AAA AAT AAC AGC CGG TCA GAA
AA:    R   E   K   L   I   E   H   Y   K   N   N   S   R   S   E

Org.: GCT CAT GTT AAA AAT AAT ATT GGT AGT AGA ACG GCT AAA AAG ACT
Opt.: GCA CAT GTC AAG AAC AAC ATT GGG TCC CGC ACC GCG AAA AAA ACT
AA:    A   H   V   K   N   N   I   G   S   R   T   A   K   K   T

Org.: GTT TCT ATA GCT TTA AAA TCC GGA AAT AAA TTT AGA AGT TTT AAA
Opt.: GTA AGC ATT GCG CTG AAA TCG GGC AAC AAA TTC CGC TCC TTT AAG
AA:    V   S   I   A   L   K   S   G   N   K   F   R   S   F   K

Org.: TCA GCT GCA AAA TTT CTT AAA TGC TCT GAA GAA CAG GTT AGT AAT
Opt.: TCG GCC GCT AAA TTT CTG AAA TGT TCT GAA GAA CAG GTT TCA AAT
AA:    S   A   A   K   F   L   K   C   S   E   E   Q   V   S   N
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**Fig.7.** ***Comparison of the original I-TevII sequence vs. Codon Optimized sequence (continued…..)***

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Org.: CAT CCA AAT GTT ATA GAT ATA AAA ATA ACA ATT CAT CCC GTC CCA
Opt.: CAC CCG AAT GTG ATT GAT ATT AAA ATT ACG ATT CAT CCT GTC CCG
AA:    H   P   N   V   I   D   I   K   I   T   I   H   P   V   P

Org.: GAA TAT GTT AAA ATA AAT GAC AAT ATC TAT AAA TCA TTT GTG GAT
Opt.: GAA TAT GTC AAA ATT AAC GAT AAT ATC TAC AAA TCA TTT GTG GAT
AA:    E   Y   V   K   I   N   D   N   I   Y   K   S   F   V   D

Org.: GCT GCT AAA GAT TTA AAA CTC CAC CCA AGT CGT ATT AAA GAT TTG
Opt.: GCG GCC AAA GAT CTG AAA CTC CAT CCG AGT CGC ATC AAA GAC CTG
AA:    A   A   K   D   L   K   L   H   P   S   R   I   K   D   L

Org.: TGT TTA GAT GAC AAT TAT CCA AAT TAC ATT GTT TCA TAT AAA CGG
Opt.: TGC CTG GAC GAC AAT TAT CCG AAT TAC ATT GTG AGC TAC AAG CGC
AA:    C   L   D   D   N   Y   P   N   Y   I   V   S   Y   K   R

Org.: GTA GAG AAG TAG
Opt.: GTG GAG AAA TGA
AA:    V   E   K   *
```

**Fig.7.** ***Comparison of the original I-TevII sequence vs. Codon Optimized sequence***

The 789bp I-*Tev*II gene flanked by *GGGCCC* (*Bsp120*I) restriction sites was ordered from (Sloning Biotechnology GmbH, Germany). The gene was provided into a standard cloning vector of the provider's pSlo1.1A with *Bsp120*I sites flanking I-*Tev*II gene. The sequence of I-*Tev*II gene was verified by the Sloning Biotechnology prior to shipment. The codon optimized I-*Tev*II gene (783bp) was isolated from the plasmid pSlo1.1A_I-*Tev*II (3786bp) (Fig.8a) by digesting it with *Bsp120*I (Fermentas) restriction enzyme. And was gel purified using Purelink$^{TM}$ Quick Gel extraction kit (Invitrogen) following the manufacturer's instructions.

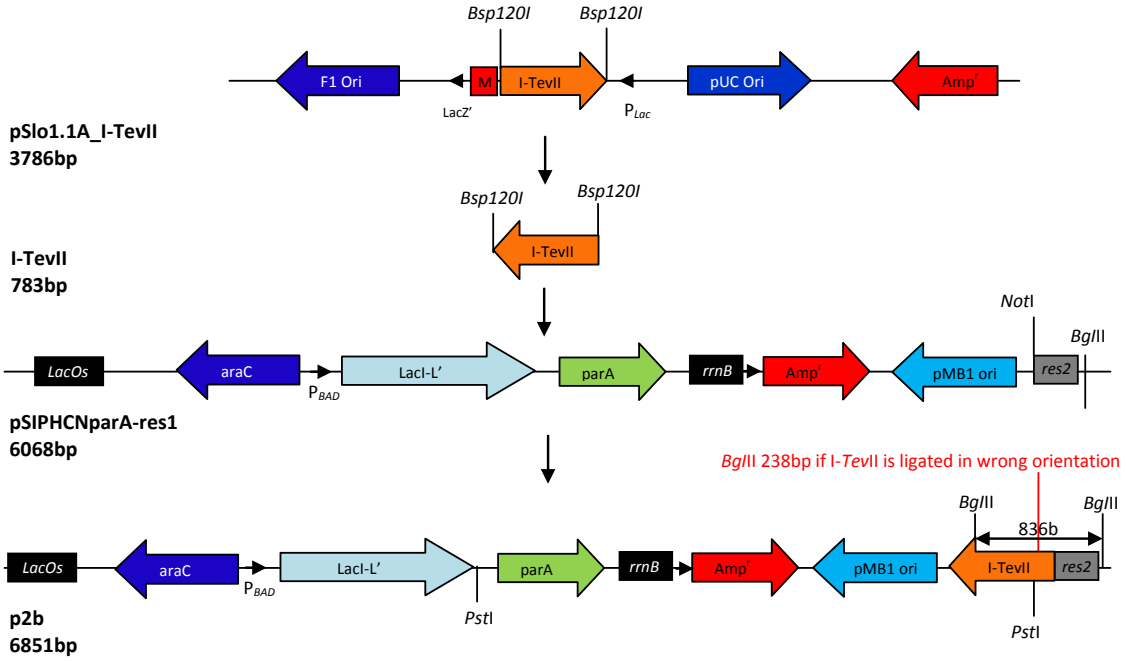



**Fig.8a.** ***Cloning strategy of plasmid p2b;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid; f1 ori, phage derived origin of replication; pUC ori, origin of replication derived from plasmid pUC19; plac, lac promoter; LacZ; encodes β-galactosidase;* I-*Tev*II, *homing endonuclease* I-*Tev*II

The plasmid pSIPHCNparA-res1 (Fig.2a) was digested with *Not*I creating 5' *GGCC* overhang which is compatible with overhangs generated through *Bsp120*I digestion of plasmid DNA. The purified I-*Tev*II gene was ligated into the corresponding sites of plasmid pSIPHCNparA-res1 using T4DNA ligase (New England BioLabs, Germany) to get the plasmid p2b (Fig.8a).

The ligation product from above cloning procedure was transformed into the *E. coli* NM522 and later into *E. coli* C2988J according to the standard molecular biological techniques by Sambrook *et.al.* [54]. almost 200 bacterial colonies from 9 different transformation procedures were picked and inoculated into 5ml Luria-Bertani (LB) medium [55] supplemented with ampicillin (100µg/ml). The plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). The samples were digested with restriction enzymes (purchased form Fermentas) listed in plasmid collection map and were loaded on 1% agarose gel (Roth$^{TM}$) stained using gel red nucleic acid gel stain (GelRed$^{TM}$ Biotium # 41003) and analyzed under UV light in a ChemiDOC$^{TM}$ machine (BioRad

laboratories) for analyzing the right clones. Several tries of cloning I-*Tev*II gene into the pSIPHCNparA-res1 turned to be unsuccessful. By knowing the toxic nature of I-*Tev*II gene due to its ability to cleave sequence other than their homing sites [40] it is therefore suspected to be a premature activation of homing endonuclease, hence the only surviving colonies were those in which I-*Tev*II gene is ligated in wrong orientation (Fig.9).

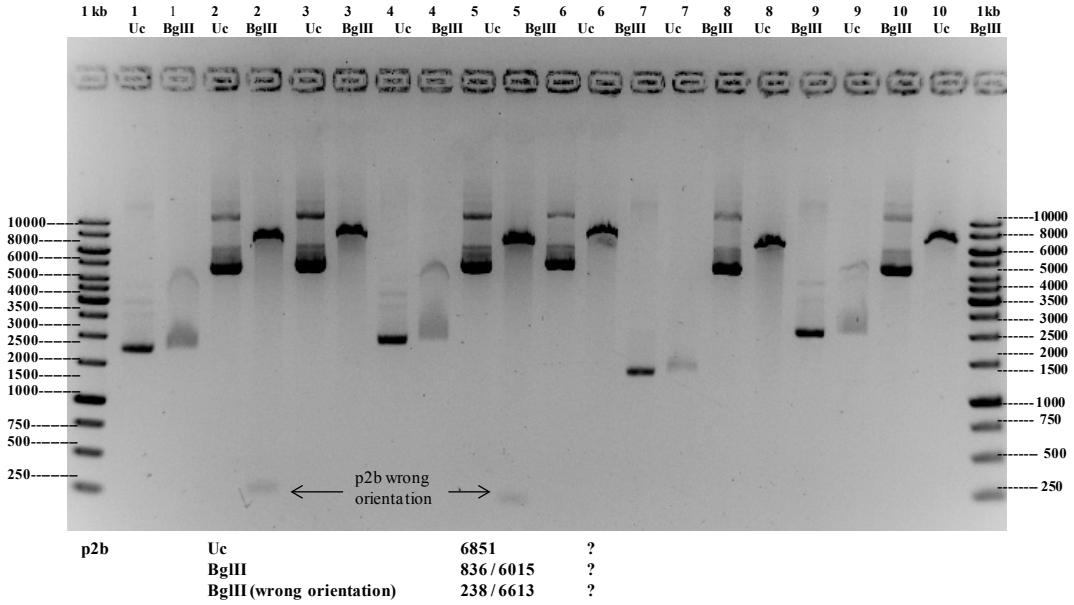



**Fig.9.** ***Restriction analysis of suggested plasmid p2b;*** *1% agarose gel showing the restriction digest of different clones of plasmid p2b(suggested) with Bgl*II *Fermentas); 1kb, GeneRuler*$^{TM}$ *1kb DNA ladder (Fermentas); clone 2 and 5 has* I-*Tev*II *in wrong orientation.*

### 1.3.4 Change of cloning strategy (pSIP-res1-pBAD)

Due to unsuccessful cloning of I-TevII gene into plasmid pSIPHCNparA-res1 the cloning strategy was changed. The new strategy included the removal of pBAD promoter from plasmid pSIPHCNparA-res1 in which the I-*Tev*II gene has to be cloned. As in final version of plasmid pSIP-I-*Tev*II the pBAD promoter is taken from already constructed plasmid p4a (Fig.4) there is no need of pBAD promoter in plasmid p2b at current stage. For this purpose the plasmid pSIPHCNparA-res1 was digested with *EcoR*V (Fermentas) which cuts the plasmid at two different positions flanking the pBAD promoter sequence along with some unnecessary parts not needed from this plasmid in construction of final plasmid pSIP-*I-Tev*II. The 1737bp fragment containing the sequences described above was removed after loading and cutting the required fragment of agarose gel the plasmid segment was gel

purified using Purelink™ Quick Gel extraction kit (Invitrogen) following the manufacturer's instructions. The plasmid was religated to get plasmid pSIP-res1-pBAD 4331bp (Fig.10).

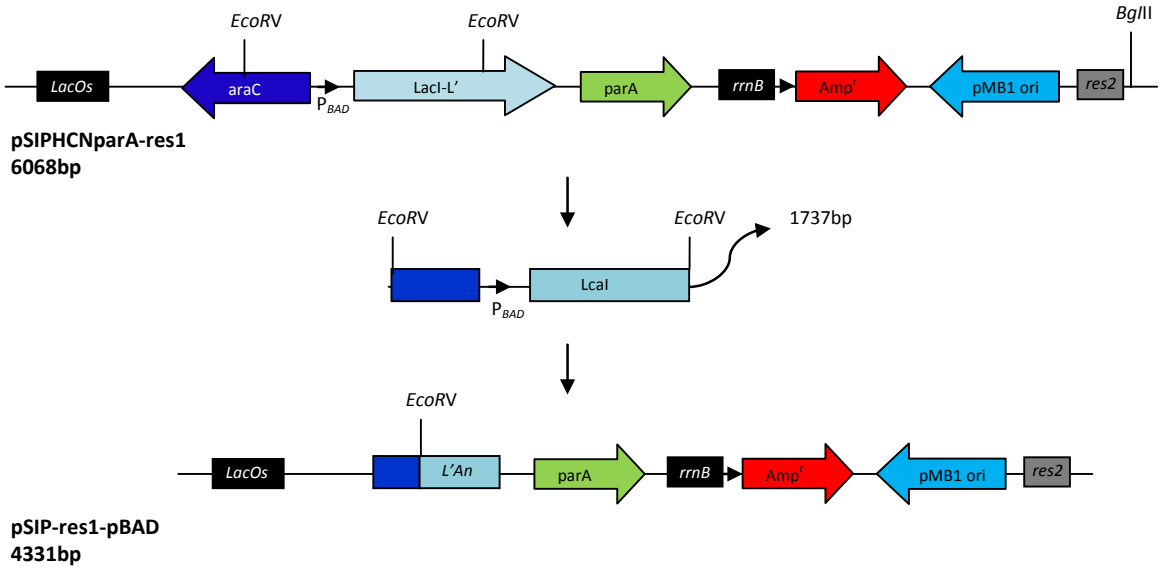



**Fig.10.** ***Cloning strategy of plasmid pSIP-res1-pBAD;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid; L'An, truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'*

The new plasmid strain *E. coli* C2992I (NEB 5-alpha F'*Iq*) (New England BioLabs) was chosen for further cloning and transformation procedures. Tight internal control of expression through $lacI^q$ makes *E. coli* C2992I ideal for cloning potentially toxic genes in them.

### 1.3.5 Construction of plasmid p2c

The new plasmid replacing the p2b was named as p2c this plasmid was constructed by digesting newly constructed pSIP-res1-pBAD (4331bp) (Fig.10) with *Not*I (Fermentas). The linearised plasmid was subjected to dephosphorelation using FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) and finally purified with PureLink™ PCR purification kit (Invitrogen) following the manufacture's protocol.

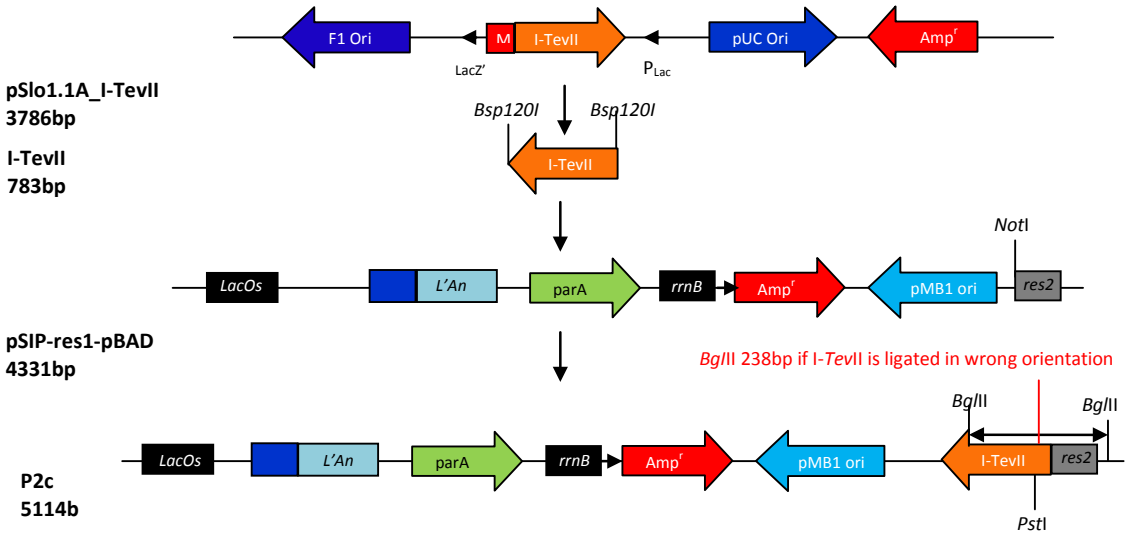



**Fig.11.** ***Cloning strategy of plasmid p2c;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$ Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid; f1 ori, phage derived origin of replication; pUC ori, origin of replication derived from plasmid pUC19; plac, lac promoter; LacZ; encodes β-galactosidase; I-TevII, homing endonuclease I-TevII; L'An, truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'*

The (783bp) I-TevII gene was isolated by Purelink™ Quick Gel extraction kit (Invitrogen) after double digesting plasmid pSlo1.1A_I-*Tev*II (3786bp) with *Bsp120*I (Fermentas) and was ligated with plasmid pSIP-res1-pBAD to get plasmid p2c (5114bp) (Fig.11). The two DNA fragments were ligated at vector to insert ratio of 1:3 using T4-DNA ligase (New England BioLabs, Germany) and transformed into *E. coli C2992I* (New England BioLabs, Germany) according to the standard molecular biological techniques by Sambrook *et.al.* [54]. 160 bacterial colonies from 8 different transformation procedures were picked and inoculated into 5ml Luria-Bertani (LB) medium [55] supplemented with ampicillin (100µg/ml). The plasmid DNA was isolated using plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). The samples were digested with restriction enzymes (purchased form Fermentas) listed in plasmid collection map and were loaded on 1% agarose gel (Roth™) stained using gel red nucleic acid gel stain (GelRed™ Biotium # 41003) and analyzed under UV light in a ChemiDOC™ machine (BioRad Laboratories) for screening of right clones(Fig.12).

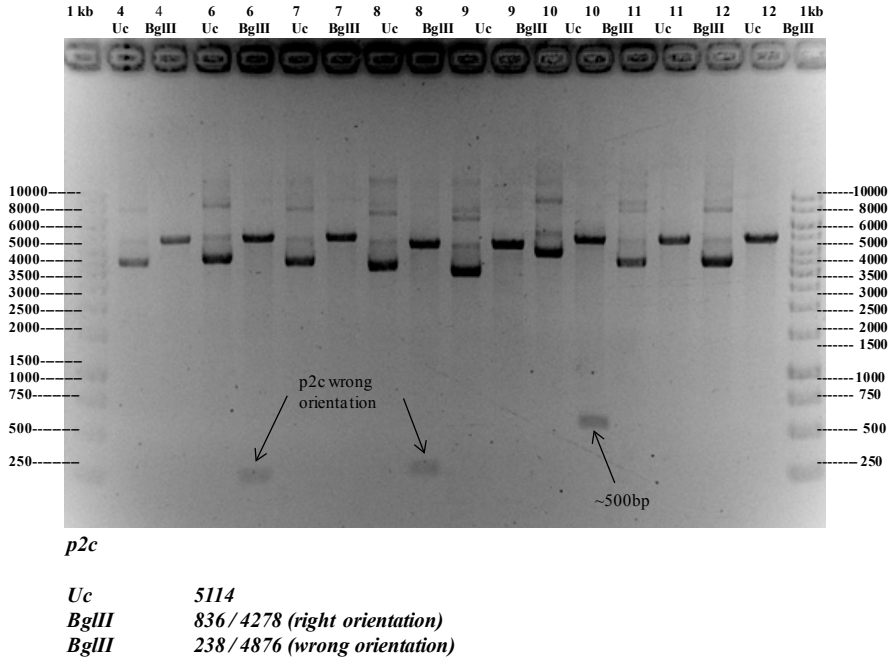



**Fig.12.** ***Restriction analysis of suggested p2c;*** *1% agarose gel picture of digested colonies of suggested plasmid p2c (*I-*Tev*II *in wrong orientation.) (clone 6 and 8), while clone 10 is has an extra Bsp120I site coming from the pSlo1.1A_I-TevII.*

Theoretic sequence of plasmid p2c having I-*Tev*II gene in right orientation with enzyme *Bgl*II will generate a 836bp and 4278bp fragment whereas plasmid p2c with I-TevII in wrong orientation will create DNA fragments of 238bp and 4876bp. However apart from above two possibilities some colonies were isolated which upon digestion with *Bgl*II showed presence of two DNA fragments around ~500bp and ~4614bp on 1%agarose gel (Fig.12). On further investigation of the theoretical sequences of the plasmids used in cloning of p2c it was found out that there was a third *Bsp120*I restriction site present in the plasmid pSlo1.1A_I-TevII which was synthesized commercially. This was cross checked by a set of restriction enzymes to locate exact position of this restriction site. It was found out that this extra *Bsp120*I site is located very close to one of the required restriction sites flanking I-*Tev*II gene making it difficult to visualize on 1% agarose gel. In order to narrow down the possible reason for unsuccessful cloning of I-*Tev*II gene into plasmid pSIP-res1-pBAD the company (Sloning Biotechnology) was requested for re-synthesize of I-TevII gene with two *Bsp120*I restriction sites. This newly synthesized I-TevII gene was received from the company in plasmid pSlo3.1A_I-*Tev*II (3351bp) with sequence confirmation showing presence of correct I-*Tev*II gene flanked by only two *Bsp120*I restriction sites.

### 1.3.6 Cloning of plasmid p2c using plasmid pSlo3.1A_I-*Tev*II

The new plasmid p2c was constructed using the I-*Tev*II gene which was isolated from the vector pSlo3.1A_I-*Tev*II (3351bp) (Fig.13). All the restriction digestion, purification of DNA samples and ligation procedures were repeated with this newly synthesized I-*Tev*II gene as explained earlier previously for construction of plasmid p2c. Approximately 60 clones were picked from 3 different transformation experiments and screened for the presence of any positive clones. This time one clone having I-TevII gene in right orientation was isolated which was hecked with four restriction enzymes listed in plasmid collection and found to be in correct orientation (Fig.14).

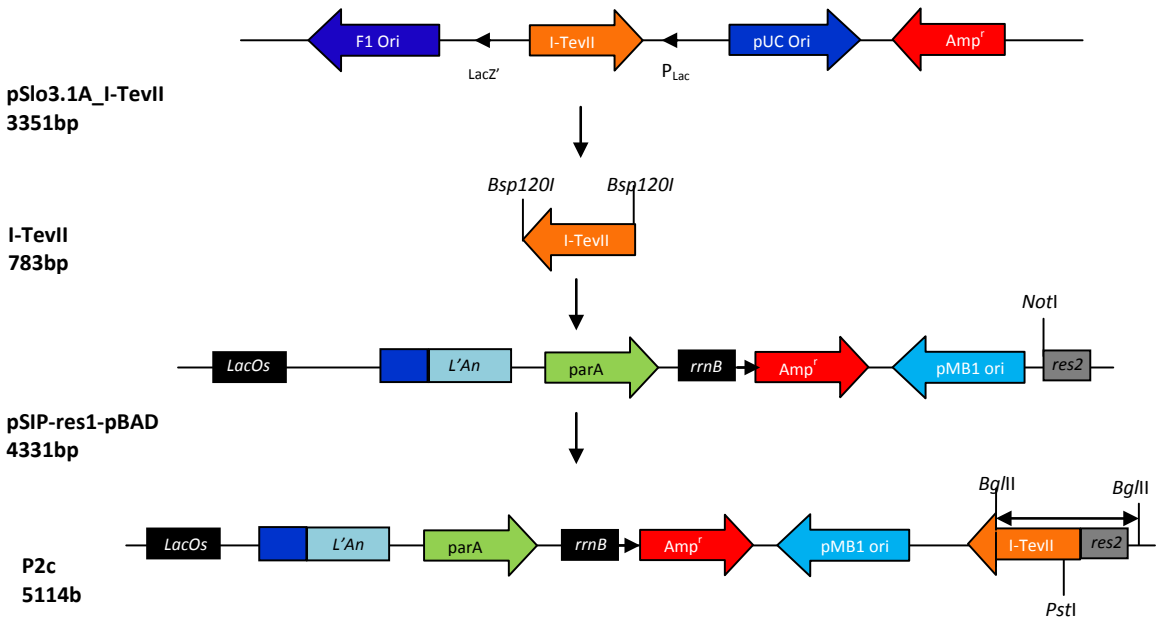



**Fig.13.** ***Cloning strategy of plasmid p2c;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid; f1 ori, phage derived origin of replication; pUC ori, origin of replication derived from plasmid pUC19; plac, lac promoter; LacZ; encodes β-galactosidase; I-TevII, homing endonuclease I-TevII; L'An, truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'*

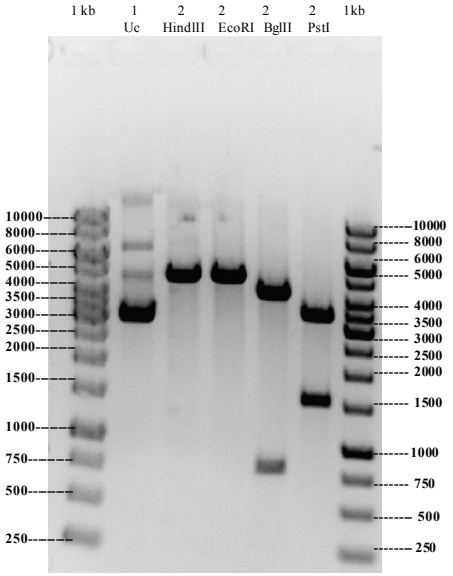



**Fig.14.** ***Restriction analysis of plasmid p2c;*** *1% agarose gel showing the restriction digest of plasmid p4a with different set of enzymes (positive clone) All enzymes (Fermentas); 1kb, GeneRuler™ 1kb DNA ladder (Fermentas)*

### 1.3.7 Construction of plasmid p5

Before proceeding for the construction of fully functional plasmid pSIP-I-*Tev*II it is necessary to construct an intermediate plasmid that carries all the characteristics of final functional plasmid except the presence of recognition sequence for I-*Tev*II. This cloning of intermediate plasmid which is named p5 is necessary for testing verifying the efficient recombination and anchoring ability of newly constructed plasmid specie. To construct plasmid p5 the two plasmids which were constructed earlier i.e. p4a (Fig.5) and p2c (Fig.13) were digested with *BspH*I and *Sal*I. The restriction product from both the plasmids was loaded on 1% agarose gel to separate the required fragments. The 4209bp fragment of plasmid p4a consisting of inverted *lacI-L'*/ parA expression cassette and 2489bp fragment of plasmid p2c consisting of antibiotic resistant gene and homing endonuclease I-*Tev*II was excised and gel purified using Purelink™ Quick Gel extraction kit (Invitrogen). The 2489bp fragment from plasmid p2c was further subjected to dephosphorelation with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) to prevent self ligation and finally purified using PureLink™ PCR purification kit (Invitrogen). The two DNA fragments were then ligated at vector to insert ratio of 1:3 using T4-DNA ligase (New England BioLabs, Germany) and transformed into *E. coli C2992I* (NEB 5-alpha F'$I^q$)(New England BioLabs) to get the bacterial colonies harboring plasmid p5 (7046bp) (Fig.14).

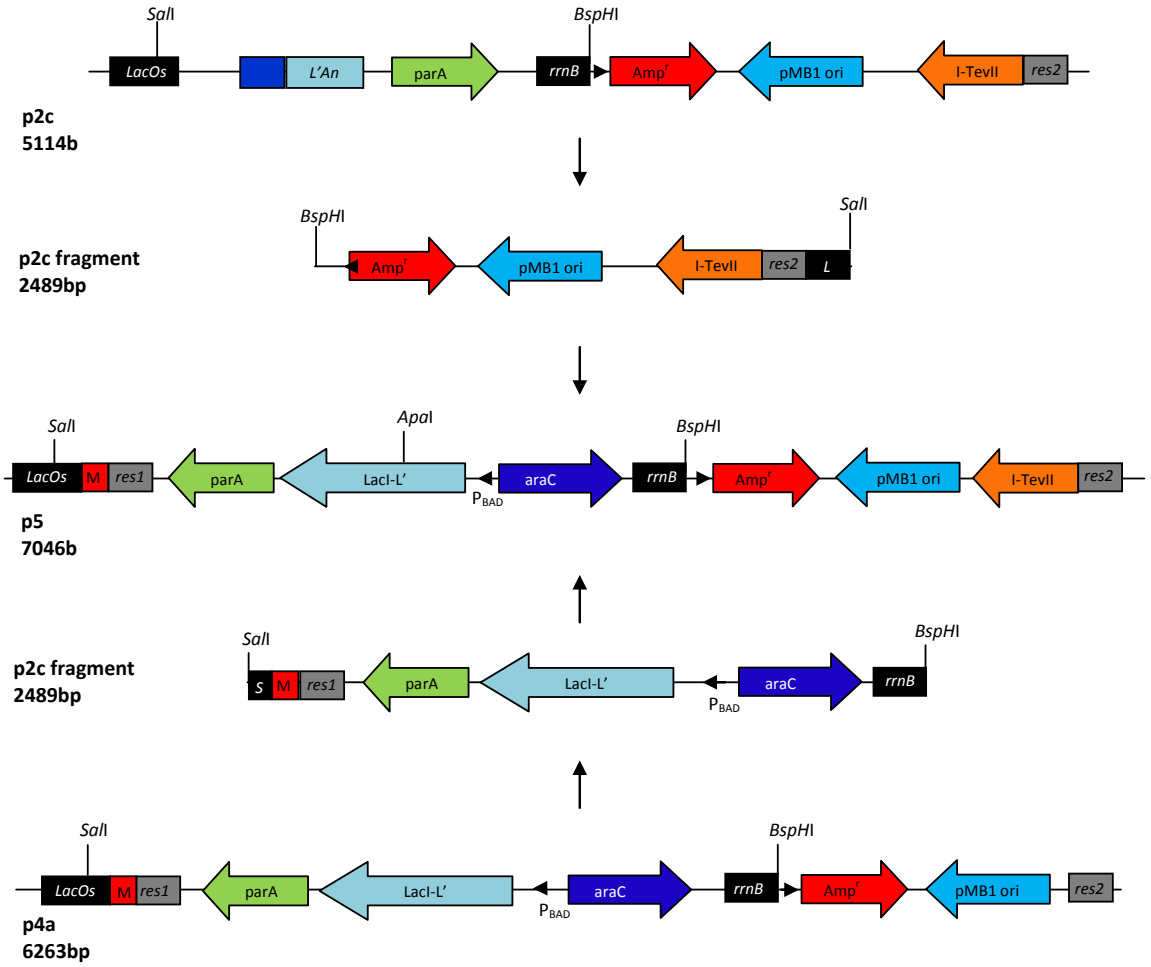



**Fig.14.** ***Cloning strategy of plasmid p5;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp^r, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid;* I-*Tev*II*, homing endonuclease* I-*Tev*II*; L'An, truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'*

Plasmid DNA isolated from 5ml culture medium of single colony freshly transformed *E. coli* C2992I harboring newly constructed plasmid p5 was subjected to restriction digesting with set of enzymes recommended for checking the plasmid accuracy based on theoretical sequence. The 1% agarose gel loaded with restriction digestion product from plasmid p5 showed an unexpected *Apa*I restriction site (Fig.15). On investigation the source of this extra *Apa*I restriction site was traced to plasmid p2c. It was further analyzed to find out its possible location within the plasmid p2c. By analyzing the theoretical sequence and double digestion of plasmid p2c with different restriction enzymes it was suspected that a point mutation G>C at position 4917nt in plasmid p2c (6852nt in case of plasmid p5) could lead to creation of extra *Apa*I site (*GGG'CCC*) this is the region where inserted gene I-*Tev*II starts. Hence it was

important that the whole region around I-*Tev*II gene should be sequenced in order to verify or check any possible amino acid change in the I-*Tev*II gene product. In the mean while the plasmid p5 was analyzed for its ability to recombine and to check the expression of lacI-L' anchor protein.

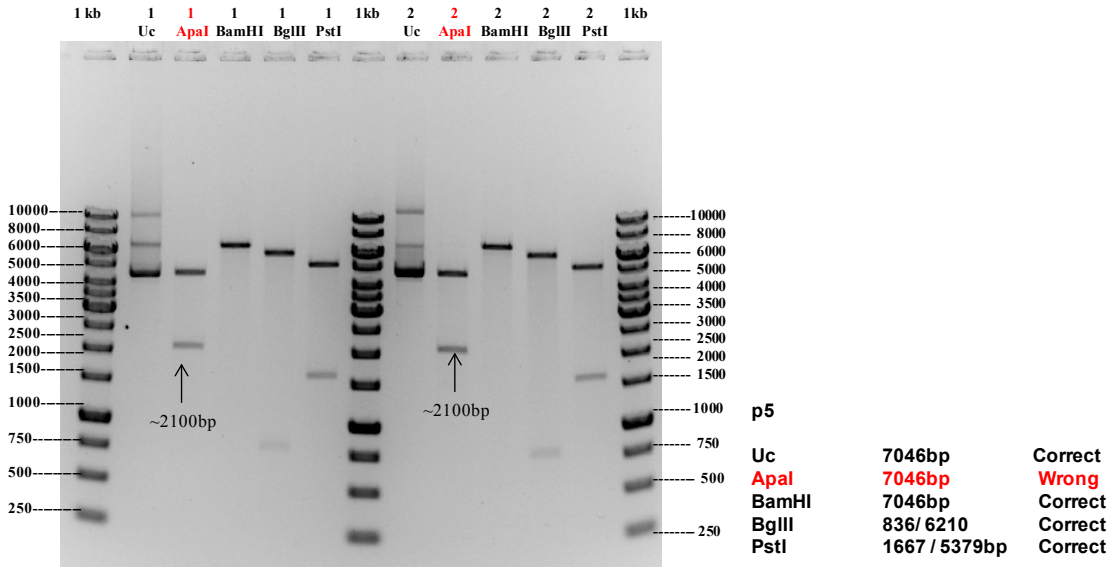



**Fig.15.** ***Restriction analysis of plasmid p5;*** *1% agarose gel showing the restriction digest of plasmid p5 with different set of enzymes showing presence of extra Apa*I *restriction site (in red) 2 different clones. All enzymes (Fermentas); 1kb, GeneRuler*$^{TM}$ *1kb DNA ladder (Fermentas)*

### 1.3.8 Protein expression study of plasmid p5

In order to check the protein expression and recombination ability of newly constructed plasmid p5. The freshly transformed *E. coli* C2992I cells with p5 and *E. coli* NM522 with plasmid p3a (control lacking recombination ability) were grown in 5ml LBv (vegetable peptone) in presence of ampicillin (100µg/ml) and 2% glucose overnight (ON) in a shaking incubator. A part of this ON culture was transferred into 100ml autoclaved nose flask containing 30ml of LBv supplemented with ampicillin 100µg/ml. and grown in 37°C water bath at continuous shaking at 300rpm. Both in plasmid p5 and p3a the lacI-L' is kept under expressional control of pBAD promoter which is induced by addition of 0.25% L-(+)-arabinose. 375µl of 20% arabinose solution is added around $OD_{600}$ nm of 0.3-0.4 which results in expression of lacI-L' fusion protein. The samples were kept under induced condition and cells were grown for another 60 min. 1ml sample each for Miniprep plasmid DNA isolation and for western blot analysis was collected at different time points before and

after the arabinose induction. The growth of cells were monitored by $OD_{600}$nm, and CFU count through plating dilutions of culture medium using automated spiral platter machine the OD and CFU values at different time points form expression study of plasmid p5 and p3a is shown in (Fig.16).

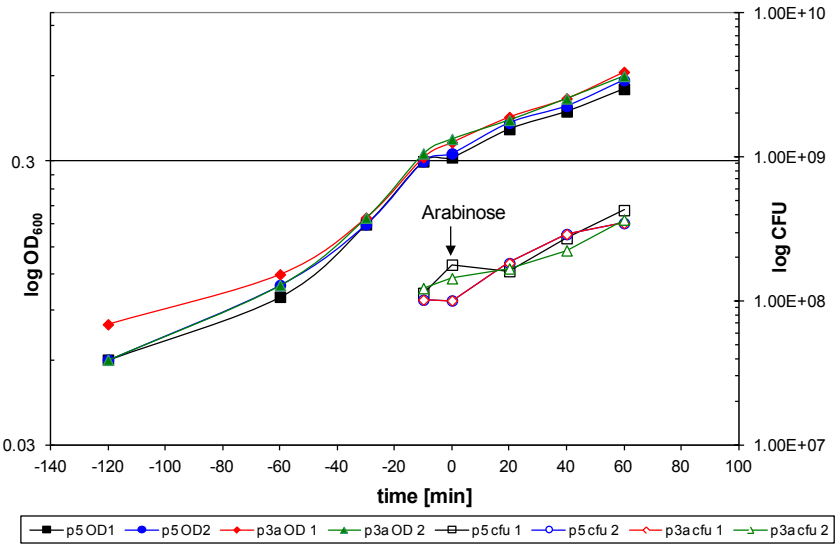



***Fig.16. Expression curve of plasmid p5;*** *OD CFU values of expression study from plasmid p5 clone 1 and 2. And plasmid p3a clone 17 and 21. No impairment seen in bacterial cell growth upon induction of pBAD promoter with addition of 0.25% L-(+)-arabinose.*

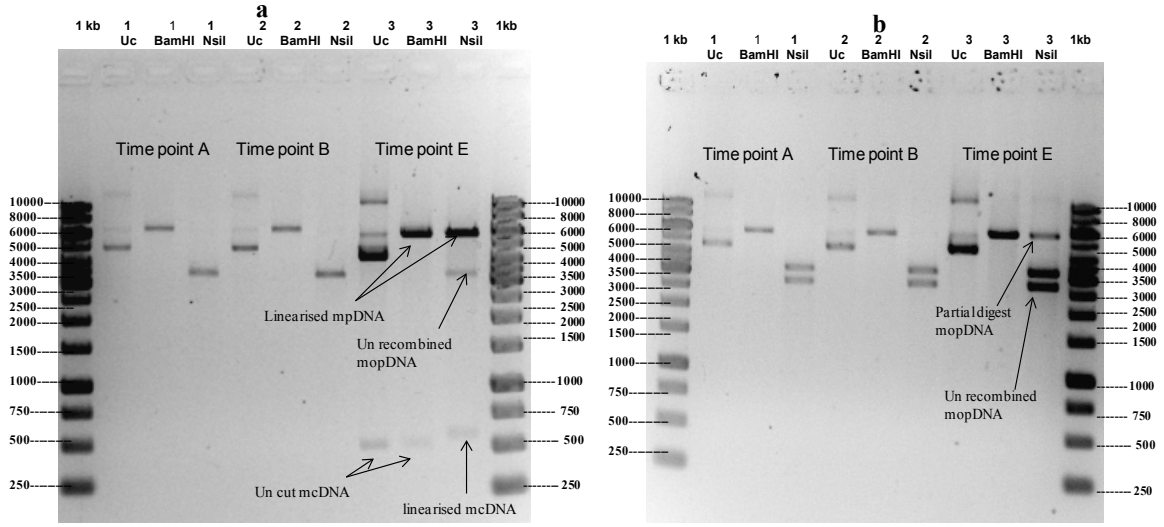



***Fig.17. Restriction analysis of recombination product of plasmid p5;*** *1% agarose gel picture* ***a)*** *recombination pattern of plasmid p5in E.coli C2988J. The cells recombine with the addition of 0.25% L-(+)-arabinose. Lane3 (Time point E digested with different enzymes), no recombination seen prior to arabinose induction (Lane 1 and 2 Time point A and B) digested with different enzymes;* ***b)*** *no recombination seen in plasmid p3a in E.coli NM522; before (Lane 1 and 2Time point A and B) and after (lane 3 Timepoint E); addition of 0.25% L-(+)-arabinose due to absence of one resolution site. Uc, uncut; BamHI, restriction enzyme; NsiI, restriction enzyme; and 1kb, GeneRuler™ 1kb DNA ladder (Fermentas)*

The recombination product of plasmid p5 and plasmid p3a which served as a control is documented on 1% agarose gel (Fig.17) The gel electrophoresis is carried out after linearizing the DNA samples with restriction enzymes that cuts once in mpDNA and once in mcDNA. The plasmid p5 is recombined upon arabinose induction into mcDNA and mpDNA the mcDNA carries only the lacOs and some spacer sequences where as the mpDNA contains the BB and antibiotic resistance cassette. No recombination is seen in samples before the addition of 0.25% L-(+)-arabinose in plasmid p5 (Fig.17, a) and in control plasmid p3a (Fig. 17, b). Western blot analysis for detection of LacI-L' fusion protein is carried out anti Lac repressor serum. The clear background expression of LacI-L' can be seen at about ~45 kDa at the beginning of time point C 20 min after induction (Fig.18, a), which is giving stronger signals at time point D and E (Fig.18, b).

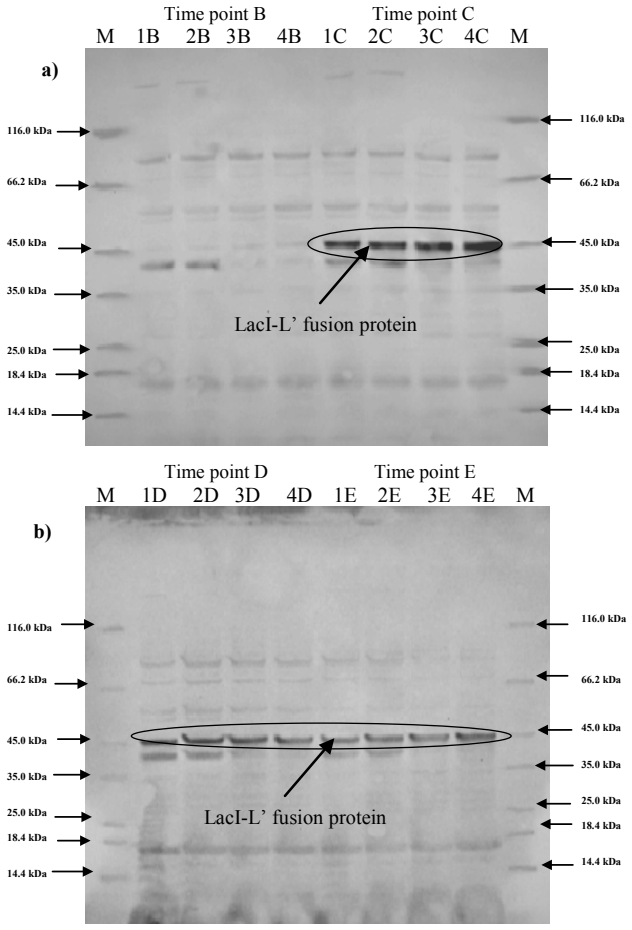



**Fig.18.** ***Western blot analysis of LacI-L';*** *Western blot analysis for detection of LacI-L' fusion protein with anti LacI antiserum in E.coli C2988J harboring plasmid p5 clone 1 and 2(lane 3b,4b,3c,4c, 3d,4d,3e and 4e) and in E.coli NM522 harboring plasmid p3a clone 17 and 21 (lane 3b,4b,3c,4c,3d,4d,3e and 4e); LacI-L' is under control of pBAD promoter which is induced by the addition of 0.25% L-(+)-arabinose at time point B;* ***a)*** *clear band of lacI-L' fusion protein seen around ~45kDa 20 min(time point C) after the addition of L-(+)-arabinose. No lacI-L' band seen at 0'min (time point B) time point of addition of L-(+) arabinose (lane 1b-4c); M=unstained protein molecular weight marker (Fermentas);* ***b)*** *clear band of LacI-L' seen at time point D and E (lane 1d-4e);M=unstained protein molecular weight marker (Fermentas)*

The clear bands at desired ~45 kDa correlates to the fact that LacI-L' was expressed by the addition of L-(+)-arabinose which was added at time point B. No expression of LacI-

L' fusion protein observed at time point B which is 0 min and the time point of arabinose addition (Fig.18, a).

### 1.3.9 Sequencing of plasmid p5 (First attempt)

In order to determine the possible effect of mutation which lead to extra *Apa*I restriction site in the plasmid p5 the sequencing was done. The plasmid p5 was transformed into *E. coli C2988J* endonuclease deficient (*endA*-) strains to get satisfactory results as required by the service providing company. The Midiprep of plasmid p5 was prepared using PureYeild$^{TM}$ plasmid Midiprep system (Promega) following the manufacturers instruction. 200µl of Midi Prep sample was sent into a screw tubes to Microsynth, CH. (Switzerland) for sequencing (primer walking service D2 verification both strand publication quality). The following sets of primers (Table.1) were designed to sequence the I-*Tev*II gene (6067-7046nt) along with the *res1* and spacer region (1-551nt). (Total sequenced region 1556bp)

**Table.1. List of primers used for sequencing of plasmid p5**

| Primers | I.D. | Sequence |
|---|---|---|
| 1Ap5F | 6702 | 5'-AGTTGGTAGCTCTTGATC-3' |
| 1Ap5R | 6703 | 5'-ACCGCATCATCACGCTTC-3' |
| 1Ap5F.2 | 6722 | 5'-TTCTTAAAGCCAATGTAG-3' |
| 1Ap5R.2 | 6740 | 5'-CCTGAAGATCGCGAACTC-3' |
| 1Ap5R.3 | 6741 | 5'-TCAAATCACCCGAATGTG-3' |

The sequencing results showed that this extra ApaI site is due to the deletion of around 27bp (frame shift) at position 6822nt-6848nt in plasmid p5 exactly at the start of I-*Tev*II gene (Fig.19).

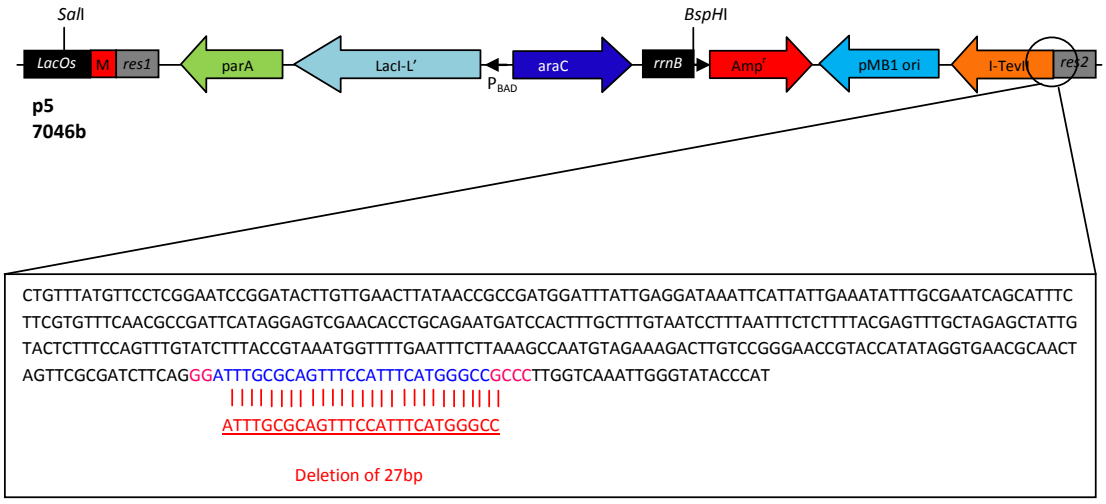


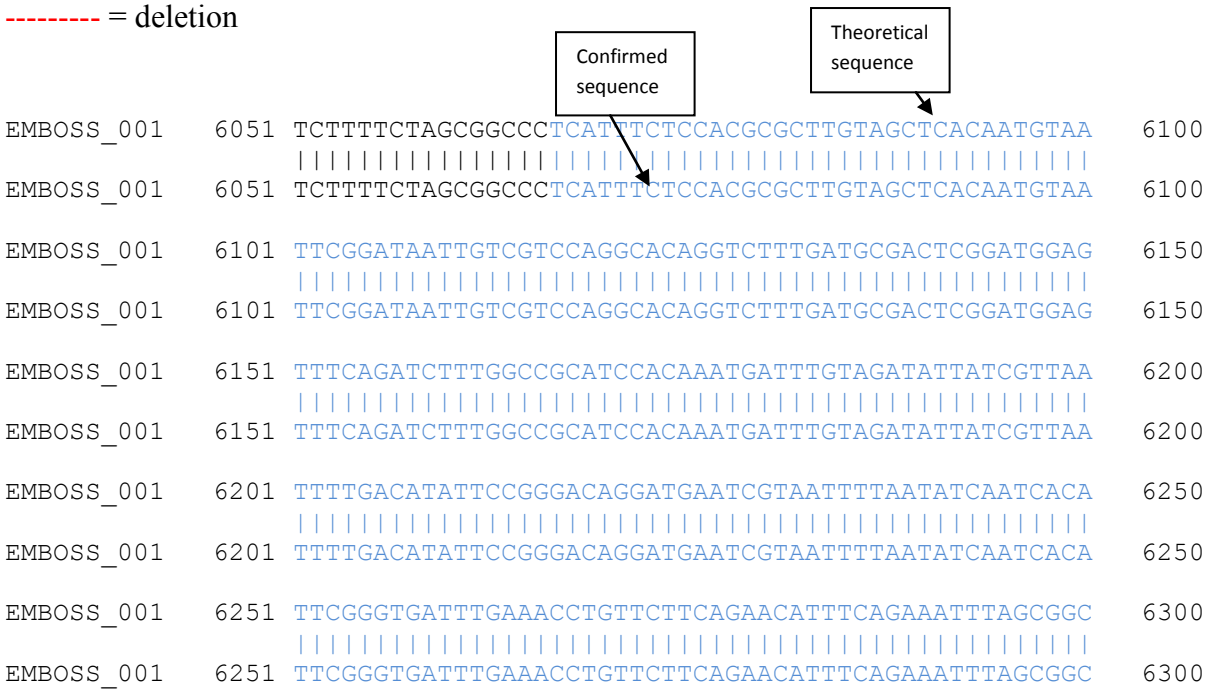

**Fig.19.** ***Schematic Drawing showing location of deletion;*** *Sequencing results for plasmid p5showing the location of deletion of 27bp (frame shift) at position 6822nt-6848nt. (newly formed ApaI restriction site marked in pink)*

Below is the sequencing result blasted against the theoretical sequence using EMBOSS Needle blast 2 sequence tool [56]. The following anomaly marked yellow was observed in the blast results. Pos 94 G instead of T. Pos 386 Insertion of C. pos 547-555 insertion of 9nt. CGG CAG TAA and deletion of 27nt at the start of I-*Tev*II gene position 6822-6848.

Light blue color= I-*Tev*II gene

⟵ = start of I-*Tev*II gene

--------- = deletion



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EMBOSS_001    6301 CGACTTAAAGGAGCGGAATTTGTTGCCCGATTTCAGCGCAATGCTTACAG   6350
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6301 CGACTTAAAGGAGCGGAATTTGTTGCCCGATTTCAGCGCAATGCTTACAG   6350

EMBOSS_001    6351 TTTTTTTCGCGGTGCGGGACCCAATGTTGTTCTTGACATGTGCTTCTGAC   6400
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6351 TTTTTTTCGCGGTGCGGGACCCAATGTTGTTCTTGACATGTGCTTCTGAC   6400

EMBOSS_001    6401 CGGCTGTTATTTTTATAGTGTTCAATCAGCTTTTCACGAATCTTATCTTT   6450
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6401 CGGCTGTTATTTTTATAGTGTTCAATCAGCTTTTCACGAATCTTATCTTT   6450

EMBOSS_001    6451 GTGTTTGAGGCTCAGAATTTTGCCCTTATGGGCGTTCGACAATTTCTGTT   6500
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6451 GTGTTTGAGGCTCAGAATTTTGCCCTTATGGGCGTTCGACAATTTCTGTT   6500

EMBOSS_001    6501 TATGTTCCTCGGAATCCGGATACTTGTTGAACTTATAACCGCCGATGGAT   6550
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6501 TATGTTCCTCGGAATCCGGATACTTGTTGAACTTATAACCGCCGATGGAT   6550

EMBOSS_001    6551 TTATTGAGGATAAATTCATTATTGAAATATTTGCGAATCAGCATTTCTTC   6600
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6551 TTATTGAGGATAAATTCATTATTGAAATATTTGCGAATCAGCATTTCTTC   6600

EMBOSS_001    6601 GTGTTTCAACGCCGATTCATAGGAGTCGAACACCTGCAGAATGATCCACT   6650
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6601 GTGTTTCAACGCCGATTCATAGGAGTCGAACACCTGCAGAATGATCCACT   6650

EMBOSS_001    6651 TTGCTTTGTAATCCTTTAATTTCTCTTTTACGAGTTTGCTAGAGCTATTG   6700
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6651 TTGCTTTGTAATCCTTTAATTTCTCTTTTACGAGTTTGCTAGAGCTATTG   6700

EMBOSS_001    6701 TACTCTTTCCAGTTTGTATCTTTACCGTAAATGGTTTTGAATTTCTTAAA   6750
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6701 TACTCTTTCCAGTTTGTATCTTTACCGTAAATGGTTTTGAATTTCTTAAA   6750

EMBOSS_001    6751 GCCAATGTAGAAAGACTTGTCCGGGAACCGTACCATATAGGTGAACGCAA   6800
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6751 GCCAATGTAGAAAGACTTGTCCGGGAACCGTACCATATAGGTGAACGCAA   6800

EMBOSS_001    6801 CTGAGTTCGCGATCTTCAGGGATTTGCGCAGTTTCCATTTCATGGGCCGC   6850
                   ||||||||||||||||||||                           ||
EMBOSS_001    6801 CTGAGTTCGCGATCTTCAGGG--------------------------GC   6823

EMBOSS_001    6851 CCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGGC   6900
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6824 CCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGGC   6873

EMBOSS_001    6901 GCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACCT   6950
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6874 GCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACCT   6923

EMBOSS_001    6951 TAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCAGATCTGGCC   7000
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6924 TAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCAGATCTGGCC   6973

EMBOSS_001    7001 ACGATGCGTCCGGCGTAGAGGATCTGCTCATGTTTGACAGCTTATC   7046
                   ||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6974 ACGATGCGTCCGGCGTAGAGGATCTGCTCATGTTTGACAGCTTATC   7019
```

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EMBOSS_001      1 ATCGATAATTGTGAGCGCTCACAATTGGAACTCAATACGACGGCAGTGAC     50
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      1 ATCGATAATTGTGAGCGCTCACAATTGGAACTCAATACGACGGCAGTGAC     50

EMBOSS_001     51 GTATCGGTGATCTTCGCAGGGATGCGAATCGAACAGCTACAGATCGTGTC    100
                  ||||||||||||||||||||||||||||||||||||||||||.|||||||
EMBOSS_001     51 GTATCGGTGATCTTCGCAGGGATGCGAATCGAACAGCTACAGAGCGTGTC    100

EMBOSS_001    101 GACGCCGGACGTGTCATATCCTGGCGAGTACGACCGGACACTGGGCGACG    150
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    101 GACGCCGGACGTGTCATATCCTGGCGAGTACGACCGGACACTGGGCGACG    150

EMBOSS_001    151 ATGACGACGTCTTCGTGACCGACGATACGCCAGAAGAACTCGAGGGGGAG    200
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    151 ATGACGACGTCTTCGTGACCGACGATACGCCAGAAGAACTCGAGGGGGAG    200

EMBOSS_001    201 TTGGTTGTCGCACCGACATCCGGCTCGATCGACGACCTCGAGCAACACCT    250
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    201 TTGGTTGTCGCACCGACATCCGGCTCGATCGACGACCTCGAGCAACACCT    250

EMBOSS_001    251 TCACGACCGGACGATCGACACGCTGACGATTCGGTTCCCACCGGATCACT    300
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    251 TCACGACCGGACGATCGACACGCTGACGATTCGGTTCCCACCGGATCACT    300

EMBOSS_001    301 CGCAGAGCAGCGAAACCTACTCCGGCACGGCTAATTGTGAGCGCTCACAA    350
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    301 CGCAGAGCAGCGAAACCTACTCCGGCACGGCTAATTGTGAGCGCTCACAA    350

EMBOSS_001    351 TTATCGATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTAC    400
                  |||||||||||||||||||||||||||||||| ||||||||||||||||
EMBOSS_001    351 TTATCGATGCATTAATTAACTAGTGAGCTCACGTG-GGCCGCCCGGGTAC    399

EMBOSS_001    401 CCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGG    450
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    400 CCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGG    449

EMBOSS_001    451 CGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACC    500
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    450 CGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACC    499

EMBOSS_001    501 TTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCTTATAA---    544
                  ||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    500 TTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCTTATAACAG    549

EMBOSS_001    545 ------GCTTTTATTTTTGCTGCTGCGCGTTCCAGGCCGCCCACACTCGT    591
                        ||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    550 CAGTAAGCTTTTATTTTTGCTGCTGCGCGTTCCAGGCCGCCCACACTCGT    599

EMBOSS_001    592 TTGACCTGGCTCGGGC    607
                  ||||||||||||||||
EMBOSS_001    600 TTGACCTGGCTCGGGC    615
```

The theoretical amino acid sequence of the I-*Tev*II gene product when compared to the proved sequence gave following results the following web based protein translation tool was used for analysis. http://web.expasy.org/translate/ The 258a.a. I-*Tev*II gene gives a

product size of 30.42kd and a charge of 31.5 assuming N and C termini are not blocked. The mutated proved sequence of 239a.a. will give protein size of 28.15kd and a charge of 26.5 assuming N and C termini are not blocked: Source http://www.encorbio.com/protocols/Prot-MW.htm.

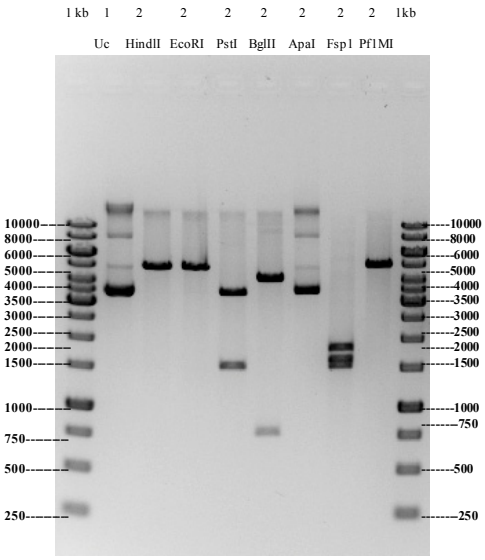
Comparison between the optimized amino acid sequence with the proved sequence. 1st attempt.

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | K | W | K | L | R | K | S | L | K | I | A | N | S | V | A | F | T | Y | M | Originalsequence |
| - | - | - | - | - | - | - | - | L | K | I | A | N | S | V | A | F | T | Y | M | Proved sequence |
| V | R | F | P | D | K | S | F | Y | I | G | F | K | K | F | K | T | I | Y | G | Deletion |
| V | R | F | P | D | K | S | F | Y | I | G | F | K | K | F | K | T | I | Y | G | * Stop codon |
| K | D | T | N | W | K | E | Y | N | S | S | S | K | L | V | K | E | K | L | K | M Start codon |
| K | D | T | N | W | K | E | Y | N | S | S | S | S | S | V | K | E | K | L | K | |
| D | Y | K | A | K | W | I | I | L | Q | V | F | D | S | Y | E | S | A | L | K | |
| D | Y | K | A | K | W | I | I | L | Q | V | F | D | S | Y | E | S | A | L | K | |
| H | E | E | M | L | I | R | K | Y | F | N | N | E | F | I | L | N | K | S | I | |
| H | E | E | M | L | I | R | K | Y | F | N | N | E | F | I | L | N | K | S | I | |
| G | G | Y | K | F | N | K | Y | P | D | S | E | E | H | K | Q | K | L | S | N | |
| G | G | Y | K | F | N | K | Y | P | D | S | E | E | H | K | Q | K | L | S | N | |
| A | H | K | G | K | I | L | S | L | K | H | K | D | K | I | R | E | K | L | I | |
| A | H | K | G | K | I | L | S | L | K | H | K | D | K | I | R | E | K | L | I | |
| E | H | Y | K | N | N | S | R | S | E | A | H | V | K | N | N | I | G | S | R | |
| E | H | Y | K | N | N | S | R | S | E | A | H | V | K | N | N | I | G | S | R | |
| T | A | K | K | T | V | S | I | A | L | K | S | G | N | K | F | R | S | F | K | |
| T | A | K | K | T | V | S | I | A | L | K | S | G | N | K | F | R | S | F | K | |
| S | A | A | K | F | L | K | C | S | E | E | Q | V | S | N | H | P | N | V | I | |
| S | A | A | K | F | L | K | C | S | E | E | Q | V | S | N | H | P | N | V | I | |
| D | I | K | I | T | I | H | P | V | P | E | Y | V | K | I | N | D | N | I | Y | |
| D | I | K | I | T | I | H | P | V | P | E | Y | V | K | I | N | D | N | I | Y | |
| K | S | F | V | D | A | A | K | D | L | K | L | H | P | S | R | I | K | D | L | |
| K | S | F | V | D | A | A | K | D | L | K | L | H | P | S | R | I | K | D | L | |
| C | L | D | D | N | Y | P | N | Y | I | V | S | Y | K | R | V | E | K | * | | |
| C | L | D | D | N | Y | P | N | Y | I | V | S | Y | K | R | V | E | K | * | | |

The protein encoded from mutated amino acid sequence of I-*Tev*II will lack 19a.a. hence the question whether this protein which is formed due to translation of this mutated I-*Tev*II gene is still functional is open, which needs to be answered by in a separate study. Little is known about the structure and working of this enzyme. The working of this shortened I-TevII gene product totally depends on its affect on proper folding of the protein and upon its presence in active site of enzyme.

### 1.3.10 Second attempt for cloning of I-TevII gene in plasmid p2c

All the cloning procedure for plasmid p2c as described in (section 1.3.6) was repeated. The absence of *Apa*I restriction site (Fig.20) in newly cloned plasmid p2c-New was confirmed before proceeding to next stage of cloning plasmid p5. The plasmid p5 was cloned as described in the (section 1.3.7) all the steps were repeated as same except the homing endonuclease carrying part is taken from newly constructed p2c-New. The newly constructed plasmid which was named p5-New (to avoid confusion) was screened for restriction sites based on theoretical sequence and was found to be correct (Fig.21). The plasmid p5-New was sent for sequencing of I-*Tev*II gene and the region surrounding both sides of the cloned insert.




| p2c-New | | |
|---|---|---|
| Uc | 5114 | Correct |
| HindIII | 5114 | Correct |
| EcoRI | 5114 | Correct |
| PstI | 1607 / 3507 | Correct |
| BglII | 836 / 4278 | Correct |
| ApaI | No cut | Correct |
| Fsp1 | 1553/1658/1903 | Correct |
| Pf1MI | 5114 | Correct |

**Fig.20.** ***Restriction analysis of p2c-New;*** *1%agarose gel picture showing restriction digest of plasmid p2c-New the enzymes marked in red are confirmatory enzymes for absence of 27 bp deletion present in p2c (first attempt). The absence of Apa*I restriction *site and presence of Fsp*1 *and Pf1M*I *suggests that the starting portion of* I-*Tev*II *gene is present. Uc, uncut; 1kb, GeneRuler*$^{TM}$ *1kb DNA ladder (Fermentas)*

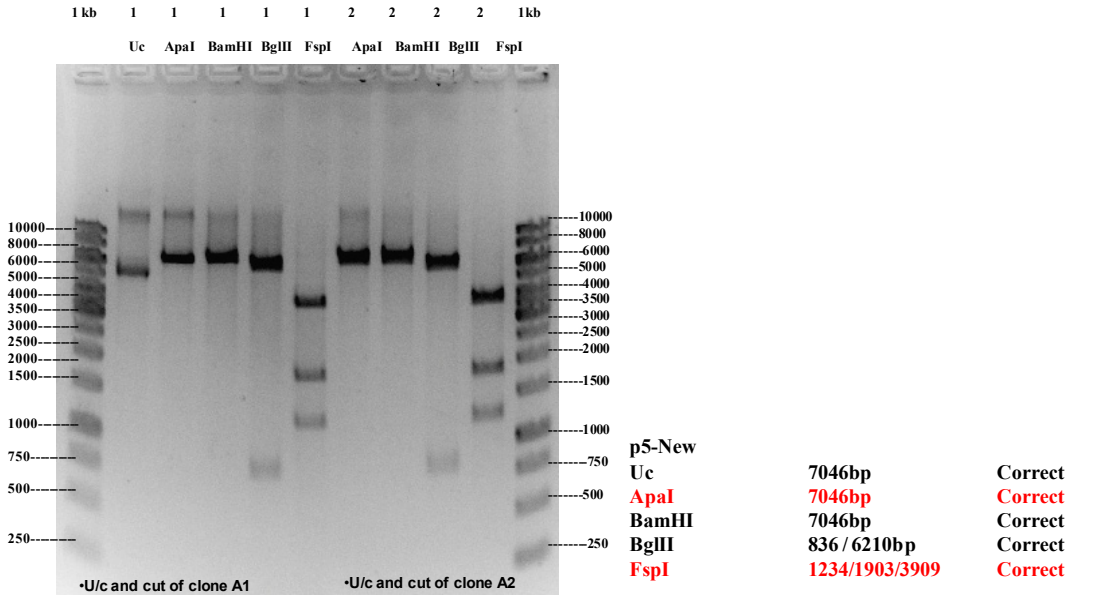



**Fig.21.** ***Restriction analysis of plasmid p5-New;*** *1%agarose gel picture showing restriction digest of plasmid p5-New; the enzymes marked in red are confirmatory enzymes for absence of 27 bp deletion previously present in p5 (first attempt). The absence of second Apa*I restriction *site and presence of Fsp*1 *suggests that the starting portion of* I-*Tev*II *gene is present in newly cloned p5-New plasmid. Uc, uncut; 1kb, GeneRuler*$^{TM}$ *1kb DNA ladder*

### 1.3.11 Sequencing of plasmid p5-New (Second attempt)

The plasmid p5-New was transformed into *E. coli* C2988J endonuclease I deficient (*endA1*) strain in order to produce better plasmid copies to get satisfactory results as required by the service providing company. The Midiprep of plasmid p5 was prepared using PureYeild$^{TM}$ plasmid Midiprep system (Promega) following the manufacturers instruction. 200µl of Midi Prep sample was sent into screw tubes to Microsynth, CH. (Switzerland) for sequencing (primer walking service D2 verification both strand publication quality). The following sets of primers (Table.1) previously designed were used to sequence the I-*Tev*II gene (6067-7046nt) along with the *res1* and spacer region (1-551nt) (Total sequenced region 2204bp). The sequencing results were blasted against the theoretical sequence using web based EMBOSS Needle blast 2 sequence tool [56]. This time the sequence results showed a 48bp deletion within the I-TevII gene at position 6647nt-6696nt in plasmid p5-New which is exactly 150bp away from the start of the gene (Fig.22).

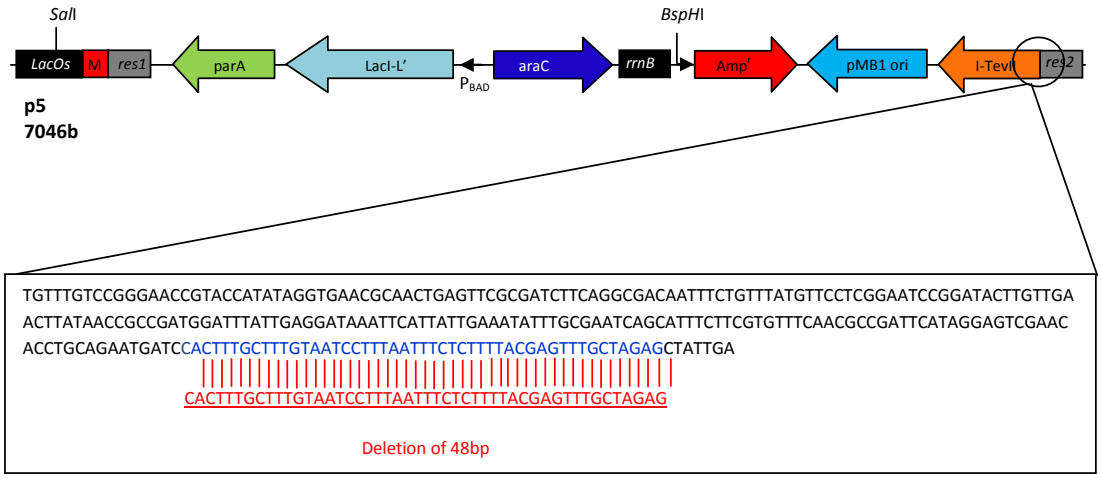


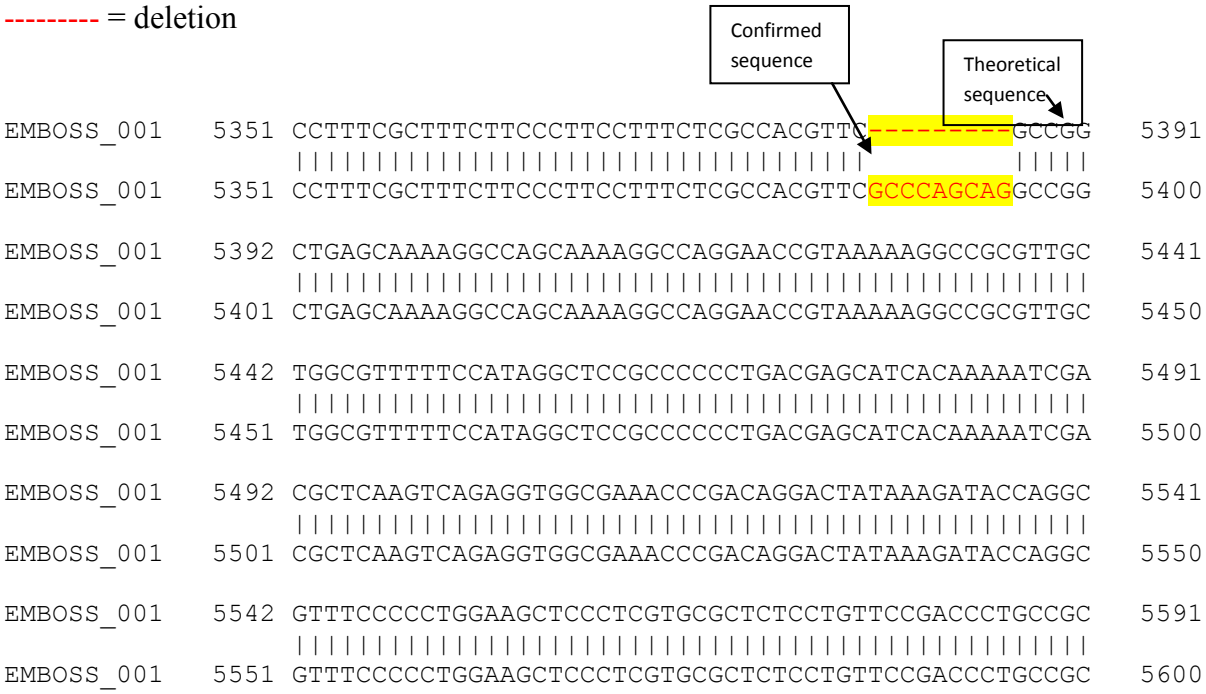

**Fig.22.** ***Schematic drawing showing the location of mutation in plasmid p5-new;*** *Sequencing data shows deletion of 48bp (no-frame shift) at position 6657nt-6704nt. 150bp from start of I-TevII gene.*

The source of this deletion was also traced back to the plasmid p2c-New suggesting the lethal nature of I-*Tev*II gene product. The following anomaly marked yellow was observed in the results. Pos 94 G instead of T. Pos 386 Insertion of C. pos 547-555 insertion of 9nt. CGG CAG TAA , insertion of 9bases at position 5388- 5396 and deletion of 48nt almost 150nt from start of I-*Tev*II gene position 6647-6696nt.

Light blue color= I-*Tev*II gene

← = start of I-*Tev*II gene

--------- = deletion



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EMBOSS_001    5592 TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT   5641
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5601 TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT   5650

EMBOSS_001    5642 CATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAA   5691
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5651 CATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAA   5700

EMBOSS_001    5692 GCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTAT   5741
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5701 GCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTAT   5750

EMBOSS_001    5742 CCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA   5791
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5751 CCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA   5800

EMBOSS_001    5792 CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG   5841
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5801 CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG   5850

EMBOSS_001    5842 TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAA   5891
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5851 TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAA   5900

EMBOSS_001    5892 CAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA   5941
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5901 CAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA   5950

EMBOSS_001    5942 GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTT   5991
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    5951 GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTT   6000

EMBOSS_001    5992 TTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG   6041
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6001 TTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG   6050

EMBOSS_001    6042 ATCCTTTGATCTTTTCTAGCGGCCCTCATTTCTCCACGCGCTTGTAGCTC   6091
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6051 ATCCTTTGATCTTTTCTAGCGGCCCTCATTTCTCCACGCGCTTGTAGCTC   6100

EMBOSS_001    6092 ACAATGTAATTCGGATAATTGTCGTCCAGGCACAGGTCTTTGATGCGACT   6141
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6101 ACAATGTAATTCGGATAATTGTCGTCCAGGCACAGGTCTTTGATGCGACT   6150

EMBOSS_001    6142 CGGATGGAGTTTCAGATCTTTGGCCGCATCCACAAATGATTTGTAGATAT   6191
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EMBOSS_001    6192 TATCGTTAATTTTGACATATTCCGGGACAGGATGAATCGTAATTTTAATA   6241
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6201 TATCGTTAATTTTGACATATTCCGGGACAGGATGAATCGTAATTTTAATA   6250

EMBOSS_001    6242 TCAATCACATTCGGGTGATTTGAAACCTGTTCTTCAGAACATTTCAGAAA   6291
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6251 TCAATCACATTCGGGTGATTTGAAACCTGTTCTTCAGAACATTTCAGAAA   6300

EMBOSS_001    6292 TTTAGCGGCCGACTTAAAGGAGCGGAATTTGTTGCCCGATTTCAGCGCAA   6341
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6301 TTTAGCGGCCGACTTAAAGGAGCGGAATTTGTTGCCCGATTTCAGCGCAA   6350
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EMBOSS_001    6342 TGCTTACAGTTTTTTTCGCGGTGCGGGACCCAATGTTGTTCTTGACATGT   6391
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6351 TGCTTACAGTTTTTTTCGCGGTGCGGGACCCAATGTTGTTCTTGACATGT   6400

EMBOSS_001    6392 GCTTCTGACCGGCTGTTATTTTTATAGTGTTCAATCAGCTTTTCACGAAT   6441
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6401 GCTTCTGACCGGCTGTTATTTTTATAGTGTTCAATCAGCTTTTCACGAAT   6450

EMBOSS_001    6442 CTTATCTTTGTGTTTGAGGCTCAGAATTTTGCCCTTATGGGCGTTCGACA   6491
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6451 CTTATCTTTGTGTTTGAGGCTCAGAATTTTGCCCTTATGGGCGTTCGACA   6500

EMBOSS_001    6492 ATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAACTTATAACCG   6541
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6501 ATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAACTTATAACCG   6550

EMBOSS_001    6542 CCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTTGCGAATCAG   6591
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EMBOSS_001    6551 CCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTTGCGAATCAG   6600

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EMBOSS_001    6601 CATTTCTTCGTGTTTCAACGCCGATTCATAGGAGTCGAACACCTGCAGAA   6650

EMBOSS_001    6642 TGATCCACTTTGCTTTGTAATCCTTTAATTTCTCTTTTACGAGTTTGCTA   6691
                   |||||
EMBOSS_001    6651 TGATC----------------------------------------------   6655

EMBOSS_001    6692 GAGCTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAATGGTTTTGAA   6741
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6656 ---CTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAATGGTTTTGAA   6702

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                   ||||||||||||||||||||||||||||||||||||||||||||||||||
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EMBOSS_001    6842 ATGGGCCGCCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGC   6891
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EMBOSS_001    6803 ATGGGCCGCCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGC   6852

EMBOSS_001    6892 CGGCATGGCGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTT   6941
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6853 CGGCATGGCGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTT   6902

EMBOSS_001    6942 TCCCGACCTTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCA   6991
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6903 TCCCGACCTTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCA   6952

EMBOSS_001    6992 GATCTGGCCACGATGCGTCCGGCGTAGAGGATCTGCTCATGTTTGACAGC   7041
                   ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    6953 GATCTGGCCACGATGCGTCCGGCGTAGAGGATCTGCTCATGTTTGACAGC   7002

EMBOSS_001    7042 TTATC   7046
                   |||||
EMBOSS_001    7003 TTATC   7007
```

```
EMBOSS_001      1 ATCGATAATTGTGAGCGCTCACAATTGGAACTCAATACGACGGCAGTGAC     50
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      1 ATCGATAATTGTGAGCGCTCACAATTGGAACTCAATACGACGGCAGTGAC     50

EMBOSS_001     51 GTATCGGTGATCTTCGCAGGGATGCGAATCGAACAGCTACAGATCGTGTC    100
                  ||||||||||||||||||||||||||||||||||||||||||.|||||||
EMBOSS_001     51 GTATCGGTGATCTTCGCAGGGATGCGAATCGAACAGCTACAGAGCGTGTC    100

EMBOSS_001    101 GACGCCGGACGTGTCATATCCTGGCGAGTACGACCGGACACTGGGCGACG    150
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    101 GACGCCGGACGTGTCATATCCTGGCGAGTACGACCGGACACTGGGCGACG    150

EMBOSS_001    151 ATGACGACGTCTTCGTGACCGACGATACGCCAGAAGAACTCGAGGGGGAG    200
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    151 ATGACGACGTCTTCGTGACCGACGATACGCCAGAAGAACTCGAGGGGGAG    200

EMBOSS_001    201 TTGGTTGTCGCACCGACATCCGGCTCGATCGACGACCTCGAGCAACACCT    250
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    201 TTGGTTGTCGCACCGACATCCGGCTCGATCGACGACCTCGAGCAACACCT    250

EMBOSS_001    251 TCACGACCGGACGATCGACACGCTGACGATTCGGTTCCCACCGGATCACT    300
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    251 TCACGACCGGACGATCGACACGCTGACGATTCGGTTCCCACCGGATCACT    300

EMBOSS_001    301 CGCAGAGCAGCGAAACCTACTCCGGCACGGCTAATTGTGAGCGCTCACAA    350
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    301 CGCAGAGCAGCGAAACCTACTCCGGCACGGCTAATTGTGAGCGCTCACAA    350

EMBOSS_001    351 TTATCGATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTAC    400
                  |||||||||||||||||||||||||||||||| ||||||||||||||||
EMBOSS_001    351 TTATCGATGCATTAATTAACTAGTGAGCTCACGTG-GGCCGCCCGGGTAC    399

EMBOSS_001    401 CCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGG    450
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    400 CCCTTGGTCAAATTGGGTATACCCATTTGGGCCTAGTCTAGCCGGCATGG    449

EMBOSS_001    451 CGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACC    500
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    450 CGCATTACAGCAATACGCAATTTAAATGCGCCTAGCGCATTTTCCCGACC    499

EMBOSS_001    501 TTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCTTATAA---    544
                  ||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001    500 TTAATGCGCCTCGCGCTGTAGCCTCACGCCCACATATGTGCTTATAACAG    549

EMBOSS_001    545 ------GCTTTTATTTTTG    560
                        ||||||||||||
EMBOSS_001    550 CAGTAAGCTTTTATTTTTG    568
```

The 258a.a. I-*Tev*II gene gives a product size of 30.42kd and a charge of 31.5 assuming N and C termini are not blocked. The mutated proved sequence of 242a.a. will give protein size of 28.55kd and a charge of 28.5 assuming N and C termini are not blocked. Source http://www.encorbio.com/protocols/Prot-MW.htm

The theoretical amino acid sequence of the I-*Tev*II gene product when compared to the proved sequence gave following results the following web based protein translation tool was used for analysis. http://web.expasy.org/translate/

Comparison between the optimized amino acid sequences with the proved sequence. 2nd attempt.

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | K | W | K | L | R | K | S | L | K | I | A | N | S | V | A | F | T | Y | M | Originalsequence |
| M | K | W | K | L | R | K | S | L | K | I | A | N | S | V | A | F | T | Y | M | Proved sequence |
| V | R | F | P | D | K | S | F | Y | I | G | F | K | K | F | K | T | I | Y | G | * Stop codon |
| V | R | F | P | D | K | S | F | Y | I | G | F | K | K | F | K | T | I | Y | G | M Start codon |
| K | D | T | N | W | K | E | Y | N | S | S | S | K | L | V | K | E | K | L | K | |
| K | D | T | N | W | K | E | Y | N | R | - | - | - | - | - | - | - | - | - | - | Deletion |
| D | Y | K | A | K | W | I | I | L | Q | V | F | D | S | Y | E | S | A | L | K | |
| - | - | - | - | - | - | I | I | L | Q | V | F | D | S | Y | E | S | A | L | K | |
| H | E | E | M | L | I | R | K | Y | F | N | N | E | F | I | L | N | K | S | I | |
| H | E | E | M | L | I | R | K | Y | F | N | N | E | F | I | L | N | K | S | I | |
| G | G | Y | K | F | N | K | Y | P | D | S | E | E | H | K | Q | K | L | S | N | |
| G | G | Y | K | F | N | K | Y | P | D | S | E | E | H | K | Q | K | L | S | N | |
| A | H | K | G | K | I | L | S | L | K | H | K | D | K | I | R | E | K | L | I | |
| A | H | K | G | K | I | L | S | L | K | H | K | D | K | I | R | E | K | L | I | |
| E | H | Y | K | N | N | S | R | S | E | A | H | V | K | N | N | I | G | S | R | |
| E | H | Y | K | N | N | S | R | S | E | A | H | V | K | N | N | I | G | S | R | |
| T | A | K | K | T | V | S | I | A | L | K | S | G | N | K | F | R | S | F | K | |
| T | A | K | K | T | V | S | I | A | L | K | S | G | N | K | F | R | S | F | K | |
| S | A | A | K | F | L | K | C | S | E | E | Q | V | S | N | H | P | N | V | I | |
| S | A | A | K | F | L | K | C | S | E | E | Q | V | S | N | H | P | N | V | I | |
| D | I | K | I | T | I | H | P | V | P | E | Y | V | K | I | N | D | N | I | Y | |
| D | I | K | I | T | I | H | P | V | P | E | Y | V | K | I | N | D | N | I | Y | |
| K | S | F | V | D | A | A | K | D | L | K | L | H | P | S | R | I | K | D | L | |
| K | S | F | V | D | A | A | K | D | L | K | L | H | P | S | R | I | K | D | L | |
| C | L | D | D | N | Y | P | N | Y | I | V | S | Y | K | R | V | E | K | * | | |
| C | L | D | D | N | Y | P | N | Y | I | V | S | Y | K | R | V | E | K | * | | |

The protein encoded from mutated amino acid sequence of I-*Tev*II will lack 16a.a. hence the question whether this protein which is formed due to translation of this mutated I-*Tev*II gene is still functional is open, which needs to be answered by in a separate study. Little is known about the structure and working of this enzyme. The working of this shortened I-TevII gene product is totally dependent on presence of this 16a.a. deletion in enzymes active site or their role in enzymes proper folding.

### 1.3.12 Alternative approach for cloning plasmid p2c (p2c-lat)

After several unsuccessful attempts to clone this lethal gene I-*Tev*II into modified version of pSIP it was only possible to get two clones from two different attempts and the

sequencing results of theses clones showed deletion mutation of 27bp and 48bp in two different parts of cloned I-*Tev*II gene.

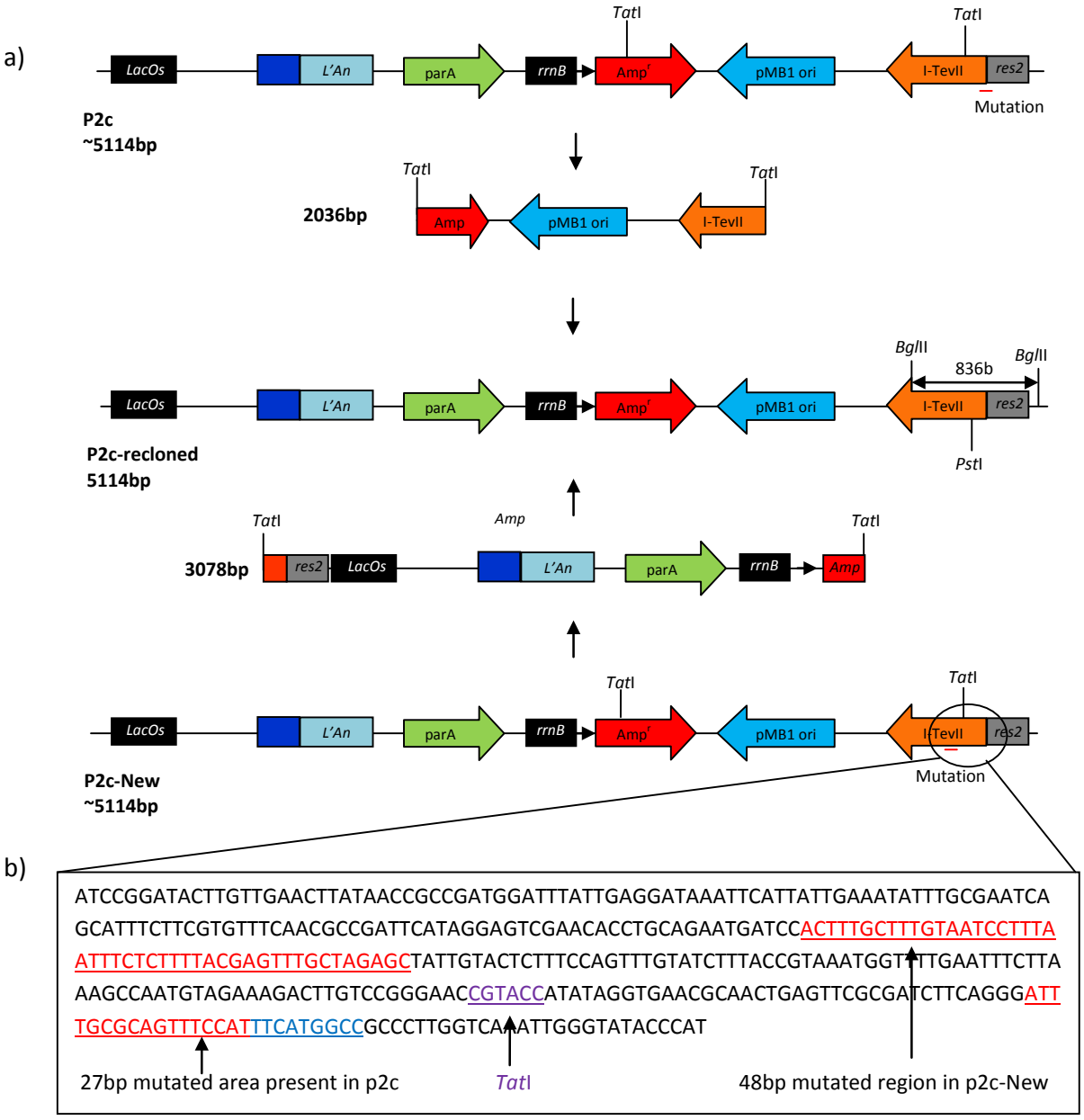



**Fig.23.** ***Alternate Cloning strategy of plasmid p2c;*** **a)** *Alternate Cloning strategy of plasmid p2c; through combining unaltered regions of* I-*Tev*II *gene from two different clones.* ***b)*** *a small part of sequence of* I-*Tev*II *gene showing location of mutation within p2c and p2c-New and presence of restriction enzyme in between them; lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid;* I-*Tev*II, *homing endonuclease* I-*Tev*II; *L'An, truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'*

By analyzing the sequencing results from these two clones i.e. p2c and p2c-New (Fig.23, b) there seemed a possibility to combine the un mutated regions of I-*Tev*II gene due to presence of a rare restriction site *Tat*I in between them. This created a possibility to get a functional plasmid that carries the whole I-*Tev*II gene without any deletions or mutations see cloning strategy (Fig.23.a). For this reason the plasmids p2c and p2c-New were digested with *Tat*I (Fermentas) and loaded on 1%garose gel. The 2036bp fragment containing the antibiotic resistance marker and the correct part of I-*Tev*II gene from plasmid p2c and 3078bp DNA segment of plasmid p2c-New was isolated using PureLink™ Quick Gel extraction kit (Invitrogen) following the manufacturers instruction. The 2036bp fragment of p2c was subjected to dephosphorelation using FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) and finally purified with PureLink™ PCR purification kit (Invitrogen) following the manufacture's protocol. The two plasmid regions were ligated using T4DNA ligase (New England BioLabs) to get the plasmid p2c-lat.

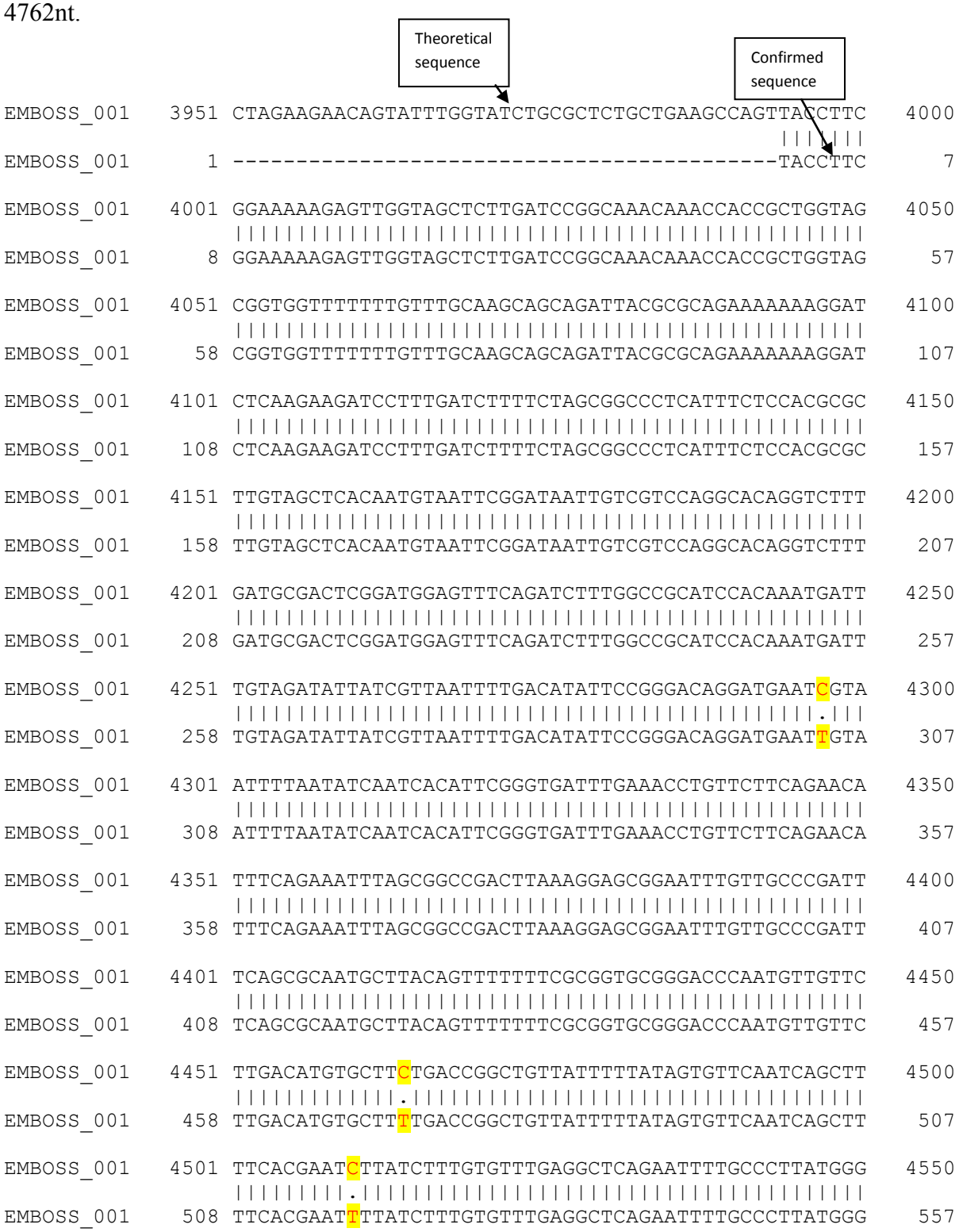
### 1.3.13 Sequencing of plasmid p2c(lat)

5 different clones of plasmid p2c(lat) was transformed into *E. coli* C2988J endonuclease I deficient (*endA1*) strain in order to produce better plasmid copies to get satisfactory results as required by the service providing company. The Midiprep of plasmid p5 was prepared using PureYeild™ plasmid Midiprep system (Promega) following the manufacturers instruction. 200µl of Midi Prep sample was sent into screw tubes obtained through this procedure were sent for sequencing to Eurofins MGW operon GmbH (Germany). The following sets of primers were designed to sequence the I-*Tev*II gene (Total sequenced region 819bp).

| Primer | location | Sequence |
|---|---|---|
| I-TevIIF | 4931-4912 | 5'-AATTTGACCAAGGGCGGCCC-3' |

The sequencing results were blasted against the theoretical sequence using web based EMBOSS Needle blast 2 sequence tool [56]. It showed 48bp deletion exact same mutation as

in plasmid p2c-New along with several other point mutations scattered along the plasmid. The following anomaly marked yellow was observed in the results. Pos 4296 T instead of C. Pos 44646 T instead of C. Pos 4510 T instead of C. Pos 4665 T instead of C. Pos 4813 T instead of C and deletion of 48nt almost 150nt from start of I-*Tev*II gene position 4715-4762nt.

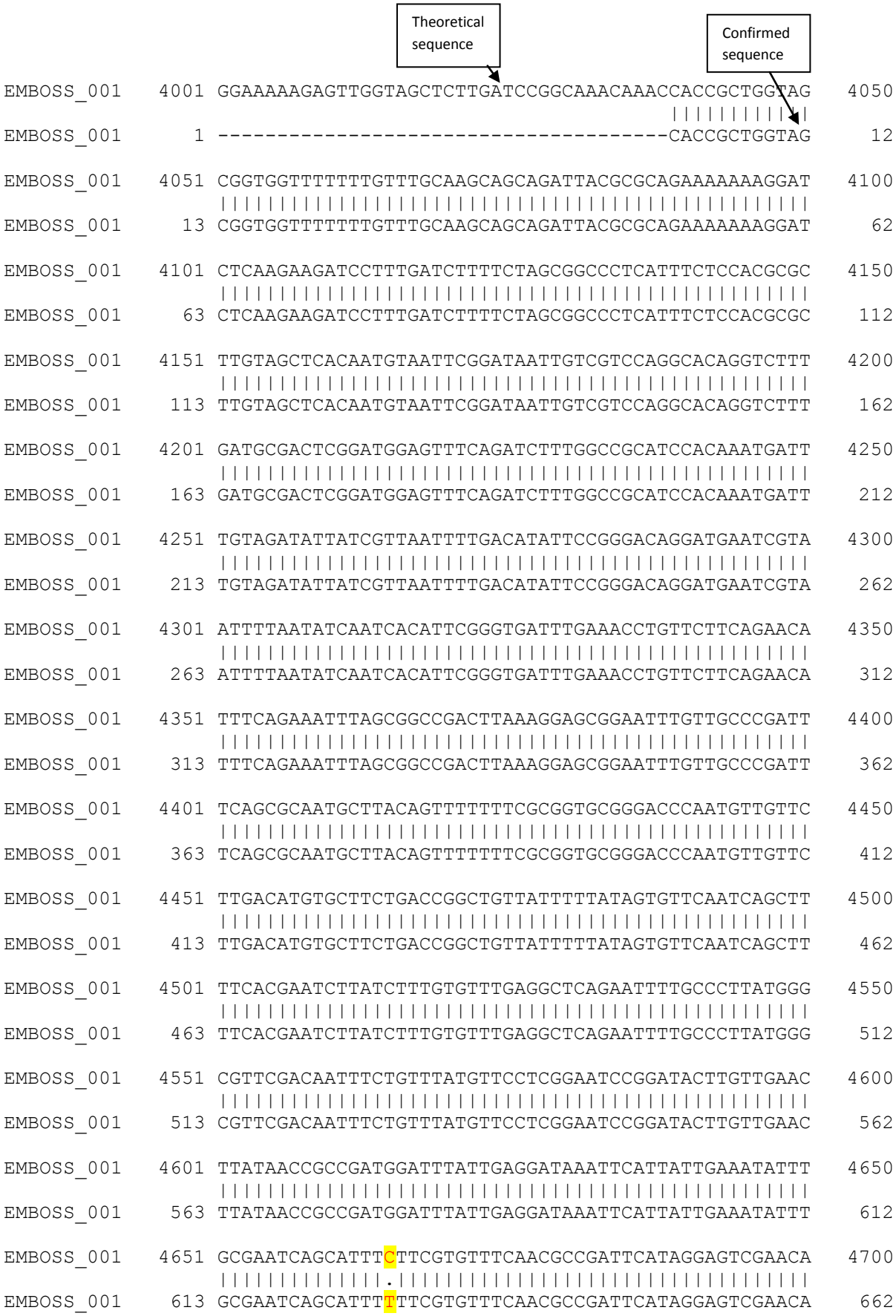


```
EMBOSS_001    4551 CGTTCGACAATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAAC   4600
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001     558 CGTTCGACAATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAAC    607

EMBOSS_001    4601 TTATAACCGCCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTT   4650
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001     608 TTATAACCGCCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTT    657

EMBOSS_001    4651 GCGAATCAGCATTTCTTCGTGTTTCAACGCCGATTCATAGGAGTCGAACA   4700
                   ||||||||||||||.|||||||||||||||||||||||||||||||||||
EMBOSS_001     658 GCGAATCAGCATTTTTTCGTGTTTCAACGCCGATTCATAGGAGTCGAACA    707

EMBOSS_001    4701 CCTGCAGAATGATCCACTTTGCTTTGTAATCCTTTAATTTCTCTTTTACG   4750
                   |||||||||||||
EMBOSS_001     708 CCTGCAGAATGATC----------------------------------    721

EMBOSS_001    4751 AGTTTGCTAGAGCTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAAT   4800
                              |||||||||||||||||||||||||||||||||||||
EMBOSS_001     722 ------------CTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAAT    759

EMBOSS_001    4801 GGTTTTGAATTTCTTAAAGCCAATGTAGAAAGACTTGTCCGGGAACCGTA   4850
                   ||||||||||||.|||||||||||||||||||||||||||||||||||||
EMBOSS_001     760 GGTTTTGAATTTTTTAAAGCCAATGTAGAAAGACTTGTCCGGGAACCGTA    809

EMBOSS_001    4851 CCATATAGGTGAACGCAACTGAGTTCGCGATCTTCAGGGATTTGCGCAGT   4900
                   ||||||||||
EMBOSS_001     810 CCATATAGGT----------------------------------------    819
```

This could be only possible in case of recloning of plasmid p2c-New. Or the survivors are only those plasmid clones that have mutated I-*Tev*II in them. The whole procedure for recloning of plasmid p2c(lat) was repeated as described in (section 1.3.6) this time also 5 clones were picked and after transformation and midiprep DNA isolation as described in previously in same section was sent to Eurofins MGW operon GmbH (Germany). The same set of primers used to sequence the I-*Tev*II gene (Total sequenced region 819bp).

| Primer | location | Sequence |
|---|---|---|
| I-TevIIF | 4986-4967 | 5’-TGCGTAATTGCTGTAATGCGC-3’ |

The sequencing results were blasted against the theoretical sequence using web based EMBOSS Needle blast 2 sequence tool [56]. It showed 48bp deletion exact same mutation as in plasmid p2c-New along with several other point mutations scattered along the plasmid. The following anomaly marked yellow was observed in the results. Pos 4665 T instead of C. Pos 4813 T instead of C and deletion of 48nt almost 150nt from start of I-*Tev*II gene position 4715-4762nt and Pos 4921 deletion of T.


Theoretical sequence
Confirmed sequence
EMBOSS_001 4001 GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAG 4050
EMBOSS_001 1 ----------CACCGCTGGTAG 12
EMBOSS_001 4051 CGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT 4100
EMBOSS_001 13 CGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT 62
EMBOSS_001 4101 CTCAAGAAGATCCTTTGATCTTTTCTAGCGGCCCTCATTTCTCCACGCGC 4150
EMBOSS_001 63 CTCAAGAAGATCCTTTGATCTTTTCTAGCGGCCCTCATTTCTCCACGCGC 112
EMBOSS_001 4151 TTGTAGCTCACAATGTAATTCGGATAATTGTCGTCCAGGCACAGGTCTTT 4200
EMBOSS_001 113 TTGTAGCTCACAATGTAATTCGGATAATTGTCGTCCAGGCACAGGTCTTT 162
EMBOSS_001 4201 GATGCGACTCGGATGGAGTTTCAGATCTTTGGCCGCATCCACAAATGATT 4250
EMBOSS_001 163 GATGCGACTCGGATGGAGTTTCAGATCTTTGGCCGCATCCACAAATGATT 212
EMBOSS_001 4251 TGTAGATATTATCGTTAATTTTGACATATTCCGGGACAGGATGAATCGTA 4300
EMBOSS_001 213 TGTAGATATTATCGTTAATTTTGACATATTCCGGGACAGGATGAATCGTA 262
EMBOSS_001 4301 ATTTTAATATCAATCACATTCGGGTGATTTGAAACCTGTTCTTCAGAACA 4350
EMBOSS_001 263 ATTTTAATATCAATCACATTCGGGTGATTTGAAACCTGTTCTTCAGAACA 312
EMBOSS_001 4351 TTTCAGAAATTTAGCGGCCGACTTAAAGGAGCGGAATTTGTTGCCCGATT 4400
EMBOSS_001 313 TTTCAGAAATTTAGCGGCCGACTTAAAGGAGCGGAATTTGTTGCCCGATT 362
EMBOSS_001 4401 TCAGCGCAATGCTTACAGTTTTTTTCGCGGTGCGGGACCCAATGTTGTTC 4450
EMBOSS_001 363 TCAGCGCAATGCTTACAGTTTTTTTCGCGGTGCGGGACCCAATGTTGTTC 412
EMBOSS_001 4451 TTGACATGTGCTTCTGACCGGCTGTTATTTTTATAGTGTTCAATCAGCTT 4500
EMBOSS_001 413 TTGACATGTGCTTCTGACCGGCTGTTATTTTTATAGTGTTCAATCAGCTT 462
EMBOSS_001 4501 TTCACGAATCTTATCTTTGTGTTTGAGGCTCAGAATTTTGCCCTTATGGG 4550
EMBOSS_001 463 TTCACGAATCTTATCTTTGTGTTTGAGGCTCAGAATTTTGCCCTTATGGG 512
EMBOSS_001 4551 CGTTCGACAATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAAC 4600
EMBOSS_001 513 CGTTCGACAATTTCTGTTTATGTTCCTCGGAATCCGGATACTTGTTGAAC 562
EMBOSS_001 4601 TTATAACCGCCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTT 4650
EMBOSS_001 563 TTATAACCGCCGATGGATTTATTGAGGATAAATTCATTATTGAAATATTT 612
EMBOSS_001 4651 GCGAATCAGCATTTCTTCGTGTTTCAACGCCGATTCATAGGAGTCGAACA 4700
EMBOSS_001 613 GCGAATCAGCATTTTTTCGTGTTTCAACGCCGATTCATAGGAGTCGAACA 662

```
EMBOSS_001    4701 CCTGCAGAATGATCCACTTTGCTTTGTAATCCTTTAATTTCTCTTTTACG   4750
                   |||||||||||||
EMBOSS_001     663 CCTGCAGAATGATC----------------------------------     676

EMBOSS_001    4751 AGTTTGCTAGAGCTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAAT   4800
                          |||||||||||||||||||||||||||||||||||||||||
EMBOSS_001     677 ------------CTATTGTACTCTTTCCAGTTTGTATCTTTACCGTAAAT    714

EMBOSS_001    4801 GGTTTTGAATTTCTTAAAGCCAATGTAGAAAGACTTGTCCGGGAACCGTA   4850
                   ||||||||||||.|||||||||||||||||||||||||||||||||||||
EMBOSS_001     715 GGTTTTGAATTTTTTAAAGCCAATGTAGAAAGACTTGTCCGGGAACCGTA    764

EMBOSS_001    4851 CCATATAGGTGAACGCAACTGAGTTCGCGATCTTCAGGGATTTGCGCAGT   4900
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001     765 CCATATAGGTGAACGCAACTGAGTTCGCGATCTTCAGGGATTTGCGCAGT    814

EMBOSS_001    4901 TTCCATTTCATGGGCCGCCCTTGGTCAAATTGGGTATACCCATTTGGGCC   4950
                   |||||||||||||||||| |||||||||
EMBOSS_001     815 TTCCATTTCATGGGCCGCCC-TGGTCAAAT--------------------    843
```

### 1.3.14 Cloning I-TevII in pET-40b (+)

It is by now understood that the I-TevII gene is difficult to clone as with every try and change in strategy a new problem arises. It is suggested in previous studies that homing endonucleases are toxic in nature because they can cleave sequences other than their homing sites [40] , therefore tight repression of expression vector is needed for their successful cloning, previously I-*Tev*II gene product has been produced in a soluble form in *E. coli* with T7 driven expression system [41, 57]. Therefore, this time the pET-40b (+) vector (Novagene) (Fig.24) was used to clone I-*Tev*II gene under expressional control of strong bacteriophage T7 promoters. The pET vector has a strong bacteriophage T7 transcription and translation signals, and expression is induced by providing a source of T7 RNA polymerase in the host cells. This external source of T7 RNA polymerase was supplied by *E. coli* C41 (Lucigen, USA). Target gene I-TevII was cloned into the MCS of pET-40b (+) Schematic diagram showing cloning strategy is shown in (Fig.24).

Unfortunately this strategy like previous one turned out to be unsuccessful. No positive clone obtained even after 3 different transformation experiments with 60 different clones are screened. The (Fig.25) shows the clone of plasmid pET-40b-I-*Tev*II in wrong orientation.

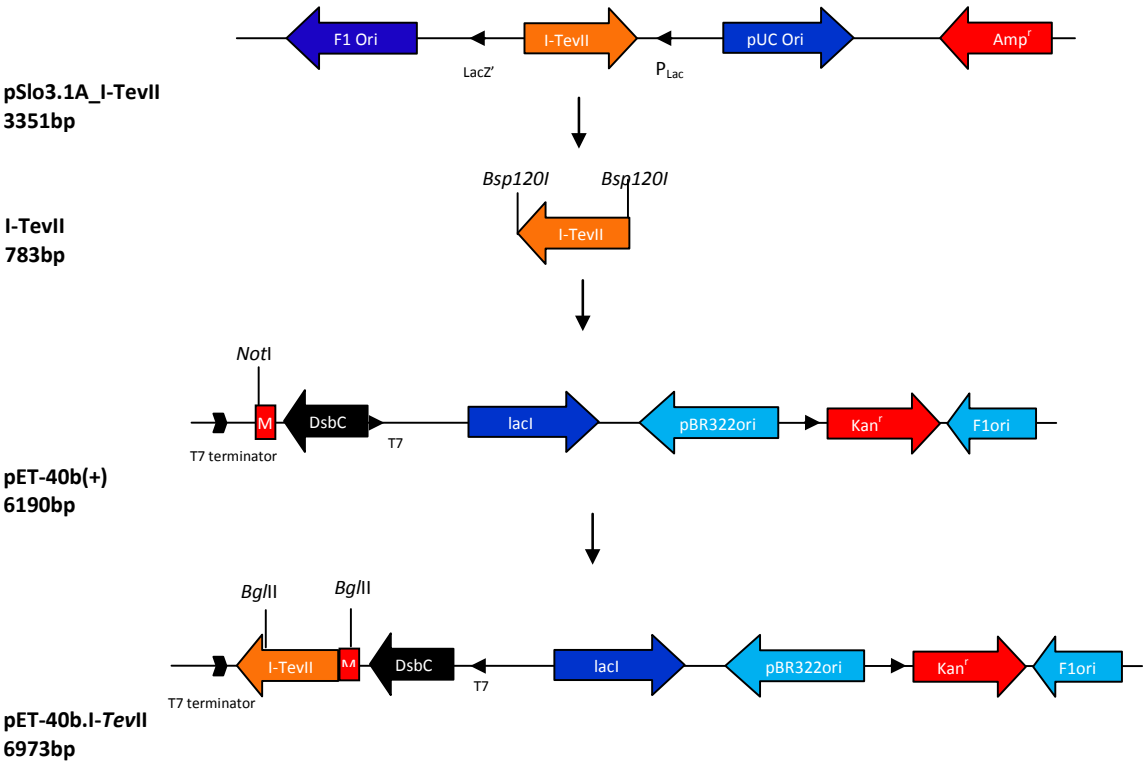



**Fig.24.** ***Cloning strategy of plasmid pET-40b.I-TevII;*** *lacI, lacI repressor;T7, bacteriophage T7 promoter; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; Kan$^r$, kanamycin resistance cassette; DsbC, Dsbc fusion protein tag. pBR322ori, origin of replication derived from pBR322 plasmid; f1 ori, phage derived origin of replication; pUC ori, origin of replication derived from plasmid pUC19; plac, lac promoter; LacZ; encodes β-galactosidase; I-TevII, homing endonuclease I-TevII; M; multiple cloning site*

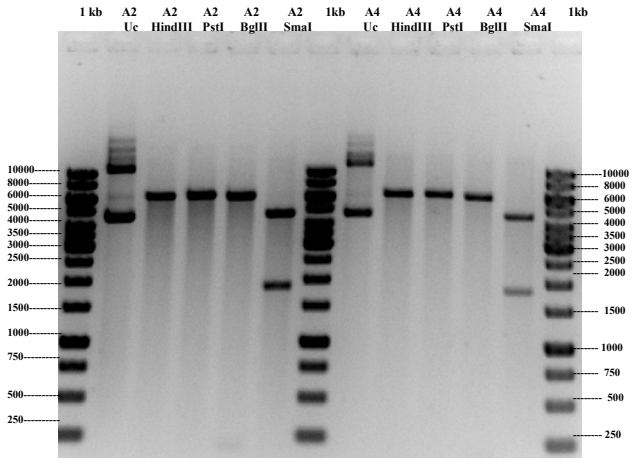



**Fig.25.** ***Restriction analysis of plasmid pET-40b.I-TevII;*** *1%agarose gel picture of restriction digest of plasmid pET-40b.I-TevII two different clones showing wrong orientation of gene I-TevII in plasmid; Uc, uncut; 1kb, GeneRuler$^{TM}$ 1kb DNA ladder*

**pET-40b.I-*Tev*II**

| | | |
|---|---|---|
| **Uc** | **6973bp** | **Correct** |
| **HindIII** | **6973bp** | **Correct** |
| **PstI** | **6973bp** | **Correct** |
| **BglII** | **781 / 6192bp** | **Wrong (orientation)** |
| **SmaI** | **2098 / 4875** | **Wrong** |

## 1.4 Discussion

The mcDNA carrying the therapeutic available gene under the control of eukaryotic promoter resulted in better transgene expression. These mcDNA are produced through different system through site specific recombination of mopDNA during which a by-product is released that are called mpDNA. These mpDNA not only leads to decrement in gene expression due to presence of unmethylated CpG motifs but also poses a serious threat for spread of antibiotic resistance gene among wild type strains. Separating these mpDNA from the mcDNA is a difficult task which is performed through cesium chloride gradient ultracentrifugation or through affinity chromatography by specific interaction of lacI protein with the lacOs sequences present on mcDNA. However, these purified mcDNA are not so immunogenic like the pathogen itself hence needs an extra help for their efficient delivery. Thus an alternate system for *in-vivo* production and loading of mcDNA was presented involving the use of Bacterial Ghost (BG) delivery technology. In this system the self immobilization plasmid was used for production of mcDNA through ParA recombination of mopDNA into mcDNA carrying lacOs site which is recognized by the membrane anchored fusion protein LacI-L'. Through this system most of the mpDNA is expelled out of the BGs through lysis tunnel however a considerable amount is still retained inside the BGs making it difficult to use these BGs in clinical trials.

In this study this particular problem was addressed and it was tried to clone and use the endonuclease activity of I-*Tev*II (which belongs to *sunY* intron of bacteriophage T4) to reduce the amount of mpDNA and mopDNA in the BGs. However, several tries in cloning this potentially toxic gene on SIP plasmid system turned out to be unsuccessful. This could be due to many possible reasons. Little available knowledge about the working of I-*Tev*II gene made it difficult to understand the reasons underlying this problem. It is therefore assumed that the I-TevII gene in combination with unknown SIP backbone sequences is lethal for the recipient bacteria, for reason, that only colonies which survived were those carrying mutated I-*Tev*II gene after the transformation.

Different bacterial strains with tight internal control of expression through $lacI^q$ have been tested in current study; these bacterial strains allow cloning of potentially toxic genes in them. Two clones with verified mutated I-*Tev*II gene at different locations have been isolated

in this study one of the mutations is frame shift however the other mutation is a deletion of 48bp sequence, this mutation results in generation of shorter protein molecule and it was assumed at that time to be non functional. Considering the time being spent on current strategy and trying different alternative path ways it was decided not to spend more time on this method and divert the focus of research on alternative ways of reducing the mpDNA in BGs which is discussed in detail in upcoming chapter II. However, it is still open to find the possible working of this shorter protein molecule being generated by mutated I-*Tev*II gene after cloning the I-TevII recognition sequence in plasmid p5 in order to get final plasmid pSIP-I-*Tev*II. The working of this shorter protein molecule generated by mutated I-*Tev*II gene depends on the possible presence of this deletion in active site or its role in proper folding of I-*Tev*II enzyme.

Another possibility is to find alternate enzymes that can replace the I-*Tev*II gene without affecting the final goal of this study. This new enzyme chosen should have more available literature and information about their working. An example of such molecule is I-*Sce*I endonuclease. This enzyme recognizes the 18bp sequence and linearises the double stranded DNA molecule which will further be degraded by the exonuclease activity of bacterial enzymes. The possibility of using *Staphylococcus aureus* nuclease A (SNUC) in reduction of mpDNA/ un-recombined mopDNA in BG preparations should also be considered as an alternate approach to endonuclease mediated reduction. In this particular case the SNUC gene can be cloned under the expressional control of pBAD promoter in addition to LacI-L'/ ParA gene in the latest an improved version of SIP plasmid p4a.

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Chapter: II

# Minicircle DNA loaded Bacterial Ghosts devoid of un-recombined mother plasmid and miniplasmid DNA sequences by nuclease activation


**Abstract**

DNA vaccination is an emerging technique currently being investigated in clinical trials. The most important concern about plasmid DNA delivery is its efficiency and safety profile. One of the safety issues has been addressed successfully by introduction of minicircle DNA (mcDNA). This mcDNA is devoid of bacterial backbone (BB) and unwanted antibiotic resistance cassette. Currently most of the mcDNA vaccines are produced in a two step process i.e. production of minicircle inside the bacterial cells, followed by the rigorous purification and separation steps. It has been shown previously that the Bacterial Ghosts (BGs) loaded with mcDNA can be produced in an efficient one step process. However, it was found that in such preparations certain amount of miniplasmid DNA (mpDNA) can still be detected in BGs. This needs to be taken care of before proceeding for use of mcDNA as a vaccine in clinical trials. Here, a new technique is reported for producing mcDNA loaded BGs devoid of unwanted DNA sequences by action of staphylococcal nuclease A *(*SNUC). Based on the small size of mcDNA and its tight attachment to the inner membrane of bacterial envelope it could be shown through real time quantitative PCR assay that a certain amount of mcDNA (2.38%) escaped the hydrolysis activity of SNUC. SNUC is more active on fragments of DNA that are larger and easily accessible i.e. mpDNA and un-recombined mother plasmid DNA (mopDNA) which are reduced to critical in mcDNA carrying BGs. BGs with mcDNA were tested in cell culture and lead to the expression of the anchored gene. It is assumed that the combination of this mcDNA immobilization along with the E-lysis and SNUC activity can represent an efficient technology for the production of *in vivo* loaded hazard free mcDNA BGs.

## 2.1 Introduction

Plasmid DNA vaccination is one of the most emerging techniques for treatment and prevention of diseases now a days. The idea of transfecting mammalian cells with plasmid DNA is well documented long ago [1]. The property of plasmid DNA as a vaccine has been investigated thoroughly since then [2-6]. However the most important issue faced currently is the development of highly safe and efficient delivery system for DNA transfer in eukaryotic cells. Currently available delivery systems are viral and non-viral. The non-viral vectors have edge over viral vectors due to their high safety profiles, relatively cheap, better capacity to deliver antigen and comparably easy to manufacture [7-12]. The plasmid DNA has two important features, the transcription unit and the bacterial backbone (BB). The former is usually responsible for the delivery of target sequence along with the necessary regulatory elements. The bacterial backbone consist of an origin of replication, antibiotic resistant gene, and unmethylated CpG motifs. These unmethylated CpG motifs are 20 times more on bacterial chromosome then on eukaryote genome due to the fact of difference in methylation pattern and frequency at which these di-nucleotides are utilized by these two different organisms [13, 14]. These CpG motifs present on BB of plasmid DNA are inflammatory in nature and triggers innate immune response [15, 16] due to which most of the DNA transfected cells are lost before they show any efficacy [17-20]. This causes serious obstacle in application of plasmid DNA used for human vaccination. Progress has been made in understanding the mechanism of episomal transgene silencing and Chen et.al. has shown that this silencing is caused due to the covalent attachment of BB to the transcription unit[21]. He also showed in his other experiments that separation of the expression cassette from the plasmid BB gives much better and higher level of transgene expression [22, 23].

On the other hand the antibiotic resistance gene poses a serious biological safety risk leading to adverse effects on patient's health [24] and responsible for the spread of antibiotic resistant strains in the environment, therefore the regulatory agencies prohibit the use of certain antibiotics in production of DNA vaccines or even emphasize on total avoidance of antibiotic resistance genes if possible [25-28]. To overcome these biological safety risks discussed above a new form of non-viral DNA delivery method comprising of only the therapeutic useful transcription unit has been developed named as mcDNA [22, 29, 30]. These minicircles not only minimized the risk of spread of antibiotic resistance gene among

wild type strain, but showed better transfection efficiency due to their reduced size [22, 30, 31].

Minicircles can be produced in *E. coli* in a different ways by site-specific recombination activity from bacteriophage λ integrase [29-31], ParA resolvase [32], *Cre* recombinase of bacteriophage P1 [33] and by the ϕC31 integrase [22]. All of the above mentioned enzymes excise the DNA sequences located between the two corresponding recombination sites (*rec*) after recognition. By placing the origin of replication and the antibiotic resistance cassette in between these two *rec* sites the mother plasmid (mop) can be divided into, a replicative miniplasmid (mp) carrying undesired sequences and the minicircle (mc) carrying only the therapeutic expression cassette [22, 29-31, 34]. Apart from the safety profile of DNA vaccines, the efficient delivery system is the main focus in designing novel drugs.

The Bacterial Ghost (BG) delivery platform is a unique and efficient method for delivering the plasmid DNA inside the target cells. BGs are non-living bacterial cell envelopes which are produced by the expression of lysis gene *E* of bacteriophage ϕX174 cloned on the plasmid [35-38]. This model is only feasible in gram negative bacteria due to their morphology of having inner membrane (IM) and outer membrane (OM). During the lysis process the transmembrane tunnel structure is formed by the fusion of IM and OM through which the cytoplasmic content is expelled out due to change in osmotic pressure of the bacterial cell and the surrounding medium [39]. The resulting bacterial envelope represents an excellent vaccine candidate for the delivery of DNA and or proteins, with an intrinsic adjuvant characteristic due to their retained structural integrity [40-51]. These BGs show high transfection efficiency for macrophages and primary dendritic cells hence, inducing higher cellular and humoral immune response in comparison to naked plasmid DNA [46, 52, 53].

The plasmid DNA delivery by immobilizing it inside the IM of BG has been established by Mayrhofer et.al. in 2005 [34]. This *in vivo* plasmid DNA packaging is based on Self Immobilization of plasmid DNA (pSIP) carrying lactose operator sites (lacOs*)* tandem repeat of a modified lactose operator sequence which is recognized by the LacI repressor protein [54, 55]. This LacI protein is fused to the hydrophobic membrane anchor (L' membrane anchor) protein derived from the lysis protein of bacteriophage phage MS2

[56]. The simultaneous expression of LacI-L' fusion gene results in the formation of a protein complex which immobilize the plasmid DNA inside the cytoplasmic membrane of *E. coli* through the hydrophobic truncated lysis protein (L') [34]. This system when combined with the mcDNA produced by ParA recombinase activity and lysis gene *E* expression resulted in self loaded mcDNA BGs [57]. In this study the eukaryotic expression cassette along with the lacOs was flanked by the two ParA resolution sites where as the recombination activity was shown close to 100%, compared to other systems described earlier by Darquet et.al. 50%, Kreiss et.al. 85% and Chen et.al. 97% [22, 29-31]. The system using ϕC31 took 2 hour to achieve 97% recombination [22] while it took only 30 min with ParA recombination system to achieve around 100% activity [57]. It has been shown that this technique of mcDNA immobilization and BG formation is efficient to get rid of most of the mpDNA however the data suggests that a small amount of mcDNA is still detectable in BG preparations [57]. Hence it is very important to develop a technique in-order to completely remove this remaining BB sequences from the BG preparations. Keeping this in view Chen et.al. in 2005 developed a technique for enzymatic digestion of mpDNA by the endonuclease activity of *I-SceI* gene present on it [58].

In a study Haidinger et.al. successfully utilized the suicidal activity of staphylococcal nuclease A (SNUC) as a secondary kill gene for producing BGs free of genetic material. [59] The thermostable nuclease A (EC 3.1.4.7) of *Staphylococcus aureus* is an extracellular enzyme [60] which cleaves the DNA and RNA into 3' phosphomononucleotides and dinucleaotides [61], this hydrolysis activity of SNUC is totally dependent on $Ca^{2+}$ ions of around 0.01M [62] while at this concentration for optimal enzymatic activity the PH of 9 to 10 is required [63] where as supplementation of 0.001M $Mg^{2+}$ ions to the above gave much higher DNA hydrolysis activity [63]. Cloned nuclease A gene of *Staphylococcus aureus* was successfully and enzymatically actively expressed in wide range of bacterial strains including gram-negative bacteria's [64-67]. It has been shown that the overexpression of this cloned gene SNUC is responsible for the loss in cell viability in different bacterial populations [68], hence the idea of using SNUC as a suicidal gene was presented [68] and this was successfully shown by Haidinger et.al. in 2003 by producing BGs devoid of genetic material by co-expression of Lysis gene *E* along with the staphylococcal nuclease A as a secondary kill gene [59].

Current study involves investigation for different possibilities in producing BGs loaded with mcDNA devoid of unwanted mpDNA / mopDNA sequences by the action of SNUC *in vivo*. Earlier it has been shown that the majority to mpDNA is lost during the production process of BGs loaded with mcDNA however a certain amount of mpDNA remains inside the BG preparations which should be removed before advancing towards the clinical application of mcDNA loaded BGs [57]. SNUC has been used in production of BGs devoid of any unwanted DNA sequences in a separate experiment [59] however it was never combined with ParA recombination for the production of mcDNA loaded BGs. It was hypothesized that the small size and strong interaction of mcDNA with the LacI-L' anchor protein through lacOs will help them escape the lethal effect of nuclease which interact and hydrolyze the larger DNA fragments i.e. mpDNA and other unwanted host DNA that are easily accessible to the enzymes. During this procedure the nuclease will be activated after the anchoring of mcDNA and completion of lysis process through addition of $Ca^{2+}$and $Mg^{2+}$ ions hence, the lethal effect of enzyme on mcDNA will be minimized as it will most probably interact and hydrolyze the DNA fragments within the viable cells and also the mpDNA molecules that are freely moving in the medium where the cytoplasmic contents of lysed cells are released after the induction of lysis gene *E*. Hence it is assumed that this combination of mcDNA, BG production and SNUC activation will provide a novel system for *in vivo* mcDNA loaded delivery vehicles that are more likely free of unwanted genetic materials eliminating any further purification steps.

## 2.2 Material and methods

### 2.2.1. Bacterial strains, plasmids and growth conditions

#### 2.2.1.1 Bacterial strains

*E. coli K12 C2988J (NEB 5-α competent cell) fhuA2 Δ (argF-lacZ) U169 phoA glnV44 ϕ80Δ (LacZ) M15 gryA96 recA1 endA1 thi-1 hsdR17)* (New England Biolabs, Germany)

*E.cloi Nissle 1917* (ARDEYPHARM Hardecke, Germany)

*E. coli K12 MC4100* [69] *F- Δ(arg-lac)U169 araD139 rpsL 150 ptsF25 fibB5301 rbsR deoC relA1*.

#### 2.2.1.2 Growth conditions

The bacterial cultivation was carried out in Luria-Bertani (LB) medium [70] with ampicillin (100µg / ml) and gentamycin (20µg / ml) when needed. The samples were supplemented with glucose at final concentration of 2%. For induction of recombination in plasmid encoding the ParA resolvase, a 0.25% L-arabinose was added at OD600 (~0.2-0.3), while for induction of synthetic *A1* promoter [71] in plasmid PGLNIc, 5mM isopropylβ-D-thiogalactopyranoside (IPTG) was used [59], The lysis is induced by the expression of cloned gene *E* on plasmid pGLNIc which is kept under transcriptional control of thermosensitive promoter / repressor system $\lambda pR_{mut}$ / *CI*857 [80] due to better lysis profile of *E. coli* Nissle 1917 at higher pH the pH of the medium was adjusted to 8.0 before the start of experiments. The SNUC was activated by addition of $CaCl_2$ and $MgCl_2$ at final concentration of 10mM and 1mM respectively [59]. Growth and lysis of the bacteria was monitored by the measuring of optical density at 600nm ($OD_{600}$), viability of cells were monitored by colony forming units (CFU) count and morphology was observed through light microscopy by making simple slides of bacterial culture.

#### 2.2.1.3 Plasmids

Plasmid pSIPHCNparA [57], pGLNIc [72], pEYFP-C1 a kind gift by Dr. Monika Sramkova NIH America (Clontech, BD-Biosciences), plasmid pSIPHCNparA-res1 (this work), p2a (this work), p3a (this work), p4a (this work), p4aEYFP-C1 (this work) p3aEYFP-C1 (this work).

### 2.2.2 Plasmid constructions

#### 2.2.2.1 Construction of plasmid p3a

To construct Plasmid p3a two intermediate plasmid constructions (pSIPHCNparA-res1, and p2a) was carried out. For easy handling, plasmid pSIPHCNparA [57] (Fig.1a) was

digested with *HindIII* (Fermentas) double cut and was re-ligated to remove the resolution site 1 (res1 140bp). The resulting plasmid was named pSIPHCNparA-res1 (6268bp) (Fig.1a) lacking recombination activity.

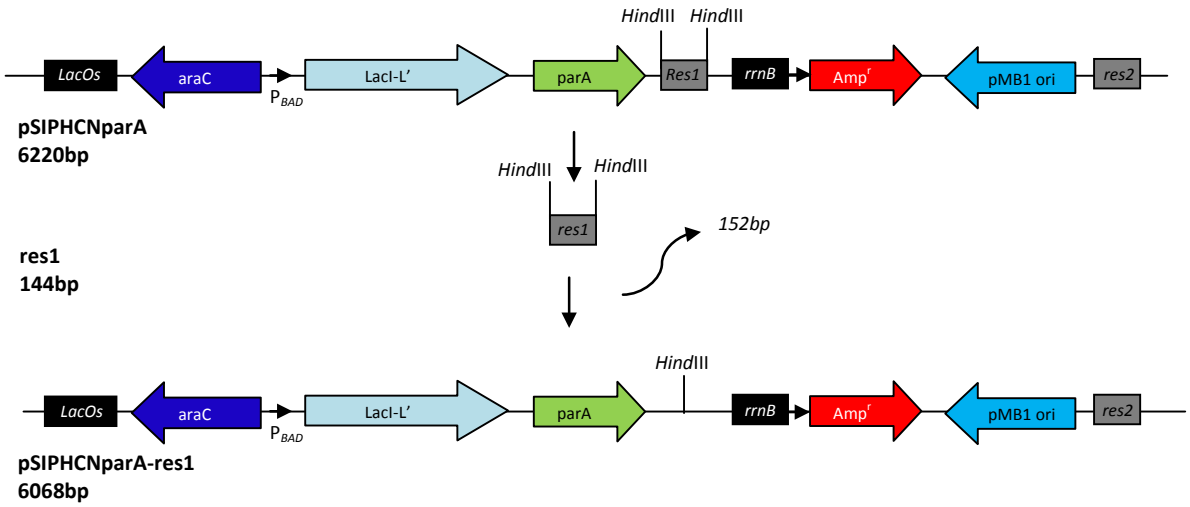


***Fig.1a. Cloning strategy of plasmid pSIPHCNparA-res1;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid*

Suitable restriction sites or multiple cloning sites MCS was introduced into this plasmid pSIPHCNparA-res1 by digesting it with *Nsi*I (Fermentas) and the 60bp MCS (Synthesized) was inserted into this site the resulting annealed plasmid is then called p2a (6128bp) (Fig.1b). This was done by mixing and incubating two primers synthesized for MCS (MCS1 and MCS2) and by introducing this annealed MCS into respective restriction site present on plasmid pSIPHCNparA-res1 using T4 DNA ligase (New England BioLabs, Germany). In order to allow the easy cloning of lethal gene (e.g. *I-Tev*II homing endonuclease that is needed for another study involving plasmid p3a) the expression cassette (LacI-L' / ParA) should be inverted for tight repression and prevention of premature expression of cloned gene. *i.e.* before recombination. For this purpose plasmid p2a was digested with *Hind*III (Fermentas) which cuts the plasmid at two different locations and the plasmid was re-ligated using T4 DNA ligase (New England BioLabs, Germany) to get plasmid p3a (6128bp) (Fig. 1c).

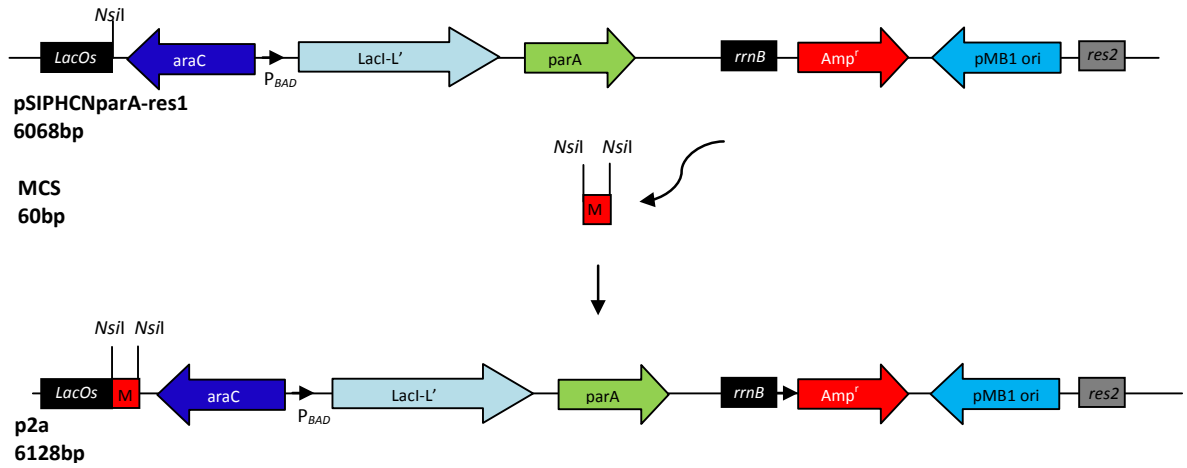


**Fig. 1b*: Cloning strategy of plasmid p2a;***
MCS1: 5'-ATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTACCTGCAGTTATAAGCTTATGCAT-3'
MCS2: 5'-TACGTAATTAATTGATCAGTCGAGTGCACGCCGGCGGGCCCATGGACGTCAATATTCGAATACGTA-3'

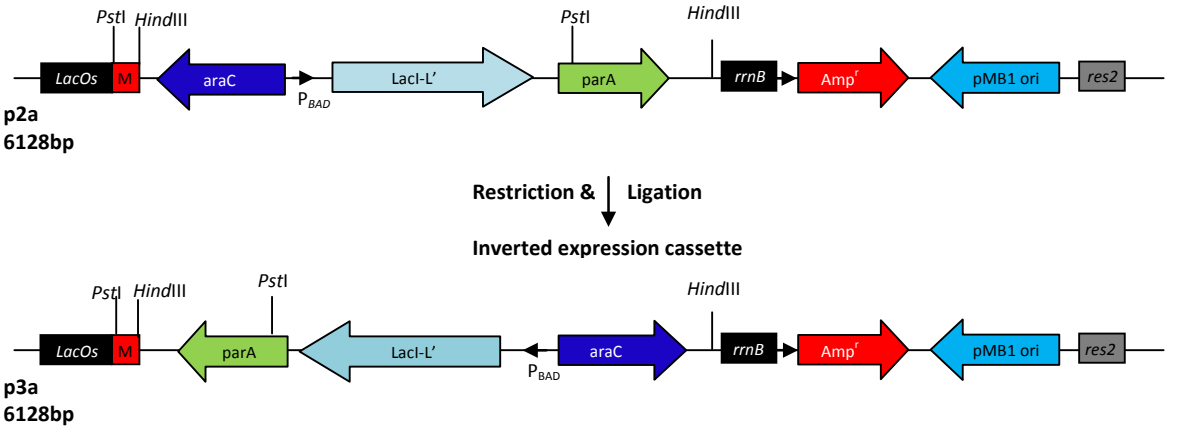


**Fig.1c. *Cloning strategy of plasmid p3a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid*

### 2.2.2.2 Construction of plasmid p4a

To get the final working plasmid p4a (which has the ability to recombine) the resolution site1 was introduced into the plasmid p3a. For this purpose the 140bp res1 site was amplified through PCR using plasmid pSIPHCNparA [57] as template and primers 5'res1K (5'– CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3') and 3res1P (5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3) (synthesized by Microsynth AG, Switzerland) with *Kpn*I and *Psi*I restriction sites (underlined sequence in primer).

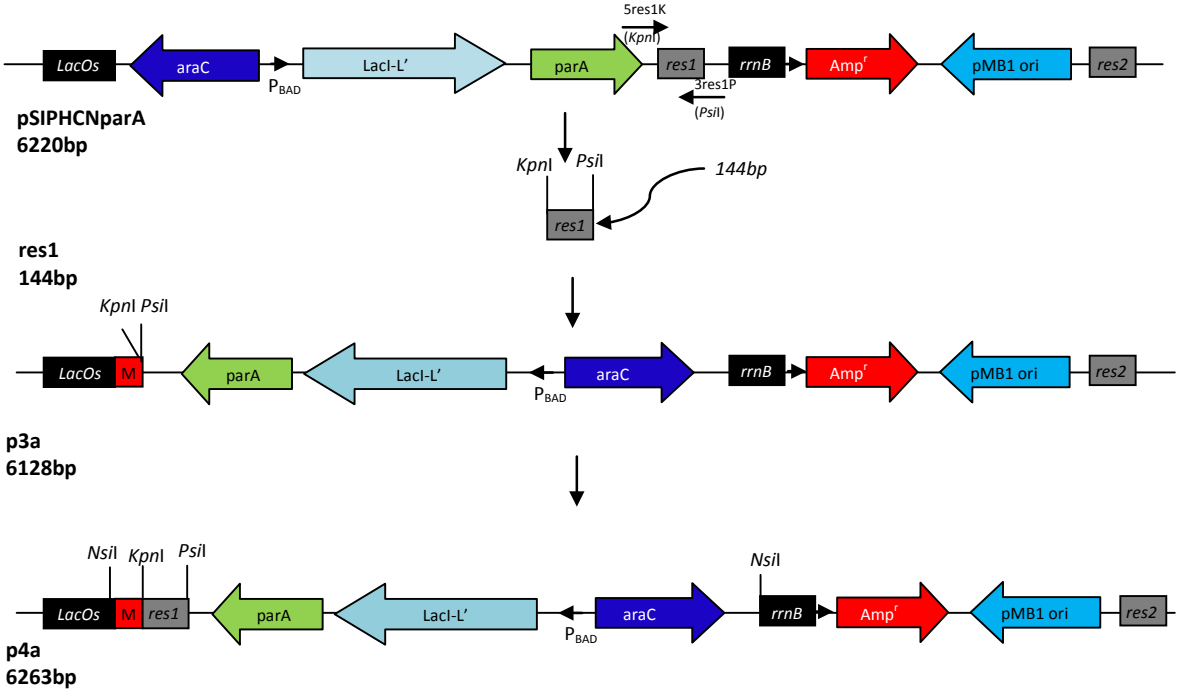


**Fig.2.** ***Cloning strategy of plasmid p4a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*5'res1K 5' – CAG CAG GGT ACC CCT TGG TCA ATT GGG GTA TAC C –3'*
*3res1P 5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3*

The PCR reaction with final volume of 25µl was carried out using 0.25µl of (50 pmol / µl) primers each, 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) DreamTaq polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U / ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95°C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95°C for 30sec, 60°C for 30sec and 72°C for 1min and final elongation of 72°C for 10 min. The PCR product was analyzed on 2% agarose gel to confirm the amplification. The PCR product was digested with *Kpn*I and *Psi*I (Fermentas) and subsequently cloned into the corresponding sites in plasmid p3a to get the vector p4a (6263bp) (Fig.2). The plasmid p4a is under control of pBAD promoter and has two resolution sites (res).

### 2.2.2.3 Construction of plasmid p4aEYFP-C1

Before constructing plasmid p4aEYFP-C1 the removal of multiple cloning site from plasmid pEYFP-C1 (4731bp) (Fig.3) a kind gift by Dr. Monika Sramkova NIH America (Clontech, BD-Biosciences) was necessary. The plasmid pEYFP-C1 was digested and *Bgl*II and *BamH*I (both Fermentas) and 51bp MCS was removed and the plasmid was religated using T4DNA ligase (New England BioLabs, Germany) to get a new plasmid pEYFP-C1-MCS (4680bp) (Fig.3a).

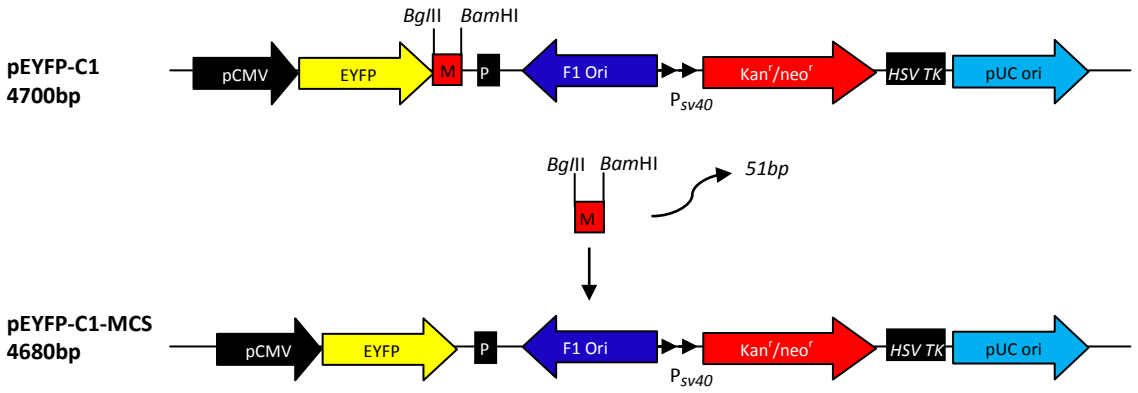


**Fig.3a.** ***Cloning Strategy of plasmid pEYFP-C1-MCS;*** *pCMV, cytomegalovirus immediate early promoter; EYFP, enhanced yellow fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; Kan$^r$/neo$^r$; Kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19.*

The 1596bp PCR fragment containing human cytomegalovirus (CMV) immediate early promoter (IE) and Enhanced yellow florescent protein (EYFP) gene along with polyA signals was amplified using primers 5'RYFP-*Sac*I 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3' and 3'RYFP-*Kpn*I 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3' (restriction sites underlined) and plasmid pEYFP-C1-MCS as template. The PCR condition was as follows. 25µl of total reaction containing 0.25µl of (50 pmol / µl) primers each (Microsynth AG), 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) Dream*Taq* polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of Dream*Taq* polymerase (Fermentas) at final conc. of (0.05U / ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95°C for 3min, as pre de-naturating temperature was used

followed by 30 cycles of 95°C for 30sec, 58°C for 30sec and 72°C for 1min and final elongation of 72°C for 10 min. The PCR product was analyzed on 2% agarose gel and subsequently digested with *Sac*I and *Kpn*I (Fermentas) and cloned into the corresponding sites in plasmid p4a resulting in vector p4aEYFP-C1 (7838bp) (Fig.3b).

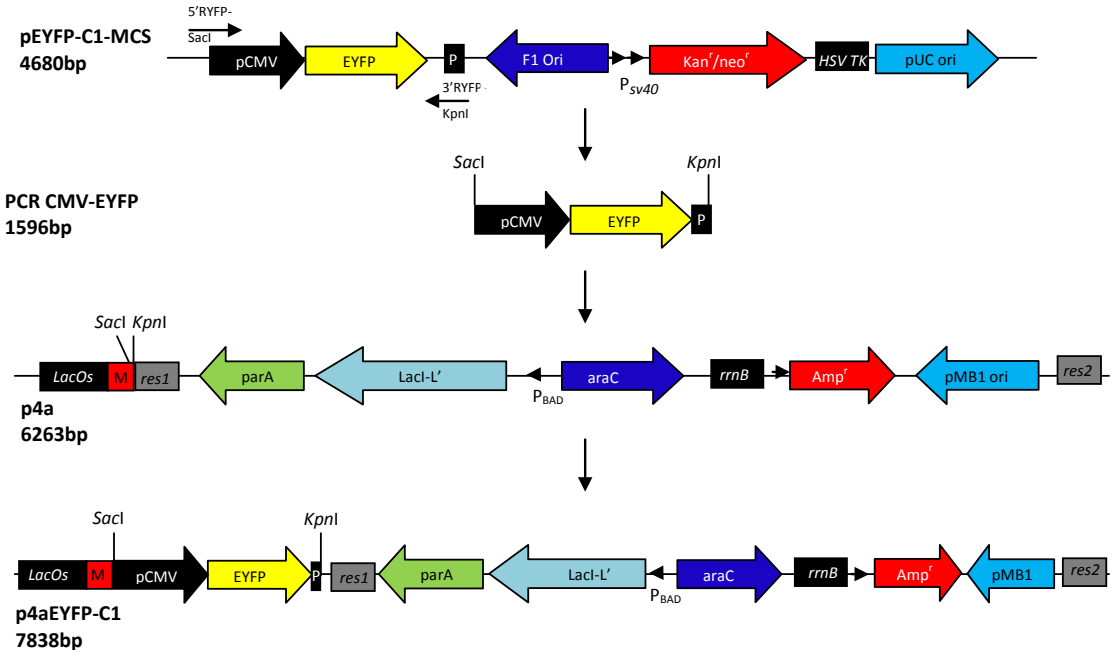


**Fig.3b.** ***Cloning strategy of plasmid p4aEYFP-C1;*** *pCMV, cytomegalovirus immediate early promoter; EYFP, enhanced yellow fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; Kan$^r$/neo$^r$; Kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 2.2.2.4 Construction of plasmid p3aEYFP-C1

For cloning plasmid p3aEYFP-C1 (7703bp) the plasmid p3a was used as a vector. The plasmid p3a was digested with *Sac*I and *Kpn*I and the 1596bp PCR fragment amplified as described above in construction of plasmid p4a was ligated together into their corresponding sites after required manipulations to get plasmid p3aEYFP-C1 (Fig.3c).

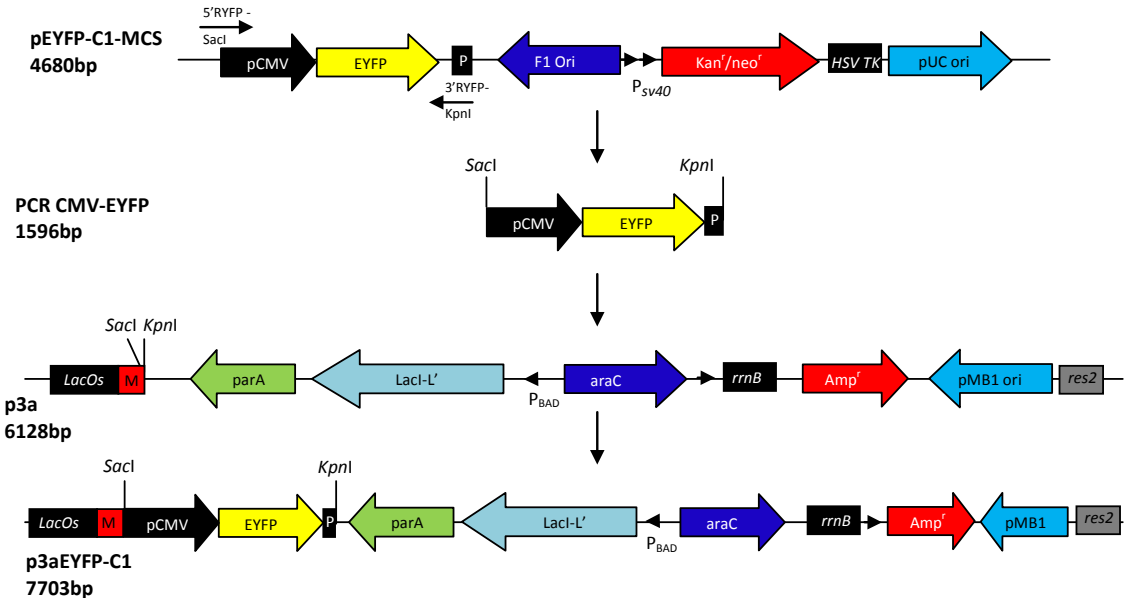


**Fig.3c.** ***Cloning strategy of plasmid p3aEYFP-C;*** *pCMV, cytomegalovirus immediate early promoter; EYFP, enhanced yellow fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; $Kan^r/neo^r$; Kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 2.2.3. Handling of DNA samples

The plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). PCR products were purified using PureLink™ PCR purification Kit (Invitrogen), while the DNA extraction from agarose gel was carried out using PureLink™ Gel extraction Kit (Invitrogen) all the above procedures were carried out following the manufacturer's protocol. All the transformation and electroporation of plasmid DNA was performed according to the standard molecular biological techniques by Sambrook et.al [73], all the restriction enzymes (unless stated separately) were purchased from Fermentas (St. Leon-Rot, Germany) and T4 DNA ligase from New England Biolabs (Frankfurt Am Main, Germany) and were used as indicated by the manufacturers / providers. PCR Amplification of the DNA fragments was carried out using iCycler iQ™ real time PCR detection system (Bio-Rad) with following DNA modifying enzymes, *Taq* DNA polymerase, Dream *Taq* DNA polymerase, *Pfu* DNA polymerase and high Fidelity DNA polymerase all provided by Fermentas (St.

Leon-Rot, Germany). Real time PCR was carried out using iQ-SYBR Green super mix (Bio-Rad) following the manufacturers instruction.

### 2.2.4 Production and purification of mcDNA and mpDNA

Bacterial strain *E. coli* MC4100 carrying plasmid p4aEYFP-C1 was grown over night at 36°C shaking, in 5ml LB supplemented with ampicillin (100µg / ml) and 2%glucose. A small part of the ON culture was added to 25ml LB supplemented with ampicillin (100µg / ml) in a nose flask. The sample was incubated in a 36°C water bath with continuous stirring 300 rpm until the $OD_{600}$ of 0.4-0.5 was achieved. The parA resolvase gene was induced by addition of 0.25% L-(+)-arabinose. And 1ml sample was collected before induction 0 min and mid phase 30 min and on 60 min for analyzing recombination pattern on 1% agarose gel. The plasmid DNA isolation was done using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). After 60 min of L-(+)-arabinose induction the cells were harvested by centrifugation at 10,000 rpm for 15min at 4°C. PureYeild™ plasmid Midiprep system (Promega) was used for isolating the plasmid DNA. The plasmid DNA was loaded on 1% agarose gel (Roth™) and the DNA was stained using GelRed nucleic acid gel stain (GelRed™ Biotium # 41003). The gel was analyzed under UV light in ChemiDOC™ machine (BioRad laboratories). The mcDNA and mpDNA fragments were excised and gel extraction was carried out using PureLink™ Gel extraction Kit (Invitrogen) finally loaded and checked on 1%agarose gel after staining with GelRed™ under ChemiDoc™ machine.

### 2.2.5 Western Blot analysis

The pellet of the bacterial cultures ~1ml is collected at different time points, immediately centrifuged at +4°C and stored at -20°C for later use. The sample was resuspended in sample buffer (Volume of 1x sample buffer (NuPAGE) in µl= $OD_{600}$ x 250) and was heated at 99°C for 10 min and finally centrifuged at 13,000 rpm for 3 min, 15µl supernatant of samples and 5µl of protein marker was loaded on 12% SDS polyacrylamide (PAA) gel [74](Invitrogen) as described by Leammli [75]. The proteins were transferred onto nitrocellulose membrane (Invitrogen) by semi-dry electro blotting [76] using xCell II Blot Module at constant volt of 60V for 30 min / (60 min when 2 gels are blotted). To identify the marker bands the nitrocellulose membrane is stained with Ponceau S (Sigma-Aldrich), and

the blocking was done overnight at +4°C with 1x Roti-Block (Roth). To detect the LacI-L' protein the sample was incubated with rabbit anti Lac repressor serum (1:5000 dilutions) purchased from Stratagene Europe, (Amsterdam, Netherlands) for 2hrs at room temperature while shaking. Followed by washing with TBST (Roth) and incubating with 1:10000 dilution of goat-derived anti-rabbit antibody linked with horseradish peroxidase (Sigma-Aldrich) for 1hrs at room temperature while shaking. Chemiluminescent detection was performed by ECL (Santa Curz kit, Amersham) and documented using BioRad ChemiDoc machine.

### 2.2.6 Cell culture and reagents

The human conjunctival cell line (Wong-Kilbourne derivative of Chang conjunctiva, CCL-20.2) was kindly provided by Prof. Bernd Binder (Medical University of Vienna, Austria) and mouse leukaemic monocyte macrophage cell line (RAW-264.7) kindly provided by Dr. Pavol kudela (Cancer Research Institute Bratislava, Slovak Republic). The cells were seeded 24 hour before transfection in a 24 well plate ($5x10^4$ cells per well) (SARATED, Ag & Co. Germany). Cells were maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 2mM glutamine (Invitrogen, Carlsband, CA), 100U / ml Penicillin (Invitrogen), 100µg / ml Streptomycin (Invitrogen), 10%FCS (Sigma Chemical Co., St. Louis, MO) and 10mM HEPES (Lonza) in a 5% $CO_2$ humidified incubator at +37°C [40]. Prior to transfection the culture medium was replaced with fresh serum free culture medium. Transfections of cells were performed using plasmid DNA (EYFP-C1, p4aEYFP-C1 and p3aEYFP-C1. 1µg / well) and transfecting reagent TurboFect™ (Thermo Scientific, Austria) following the manufacturer's instructions. Transfection efficiency of BGs carrying mcDNA (mcEYFP) produced by the Recombination Lysis and SNUC Test (RLST) was carried out in RAW-264.7 cells. Cells incubated with or without plasmids / BGs and without transfecting reagent served as negative control. Transfection efficiencies were analyzed after 24 and 48 hours of transfection by light fluorescence microscopy (Zeiss Axiovert S100 inverted microscope, Carl Zeiss Jena GmbH, Germany).

### 2.2.7 Residual free mcDNA production and immobilization inside the BGs

The bacterial strain *E. coli* Nissle 1917 carrying plasmid pGLNIc [72] and the vector p4aEYFP-C1 or p3aEYFP-C1 respectively, was inoculated and grown in 5ml LB

supplemented with antibiotics ampicillin and gentamycin at different concentrations (100µg / ml and 20µg / ml respectively) along with 2% glucose in a rotating wheel at 36°C over night (ON). Small portion of this ON culture was inoculated in a nose flasks containing 30ml LB pH 8.0. The sample was supplemented with ampicillin (100µg / ml and gentamycin (20µg / ml) but no glucose. The nose flask was kept in 36°C water bath at continuous stirring of 300 rpm with magnetic stirrer to ensure the proper aeration and equal distribution of culture medium for efficient growth. Around $OD_{600}$ of 0.2-0.3 the expression of LacI-L' fusion protein along with ParA resolvase gene was induced by adding 0.25% L-arabinose to the medium and growth was continued at same temperature for another 30 min the induction of SNUC gene was carried out by the addition of 5mM isopropylβ-D- thiogalactopyranoside (IPTG) and grown for another 30 min to accumulate enough amount of nuclease inside the bacterial cells. Lysis gene *E* was induced with the temperature up shift to 42°C and after 60 min of lysis the SNUC was activated by the addition of $MgCl_2$ and $CaCl_2$ at final concentrations of 1mM and 10mM respectively. This whole process of growth, lysis and killing of cells with SNUC was monitored by Optical density $OD_{600}$ measurement, physical observation through light microscopy and determination of CFU for viable cells through platting dilution of culture medium on LB agar plates.

### 2.2.8 Alternate protocol for production of residual free mcDNA immobilized inside the BGs

Plasmids p4aEYFP-C1 and pGLNIc [72] was co-transformed in *E. coli* Nissle 1917 and was inoculated and grown in 10ml LB supplemented with antibiotics ampicillin and gentamycin at different concentrations (100µg / ml and 20µg / ml respectively) along with 2% glucose in rotating wheel at 36°C over night (ON). Small portion of this ON culture was inoculated in a nose flasks containing 30ml LB pH 8.0. The sample was supplemented with ampicillin and gentamycin but no glucose. The nose flask were kept in 36°C water bath at continuous stirring of 300 rpm to ensure the proper aeration and equal distribution of culture medium for efficient growth. Around $OD_{600}$ of 0.2-0.3 the expression of LacI-L' fusion protein along with ParA resolvase gene was induced by adding 0.06% L-arabinose to the medium and growth was continued at same temperature for another 60 min after which the lysis gene *E* was induced with the temperature up shift to 42°C and was continued for 60 min. Finally the induction of SNUC gene was carried out by the simultaneous addition of 5mM

isopropylβ-D- thiogalactopyranoside (IPTG) and $MgCl_2$ and $CaCl_2$ at final concentrations of 1mM and 10mM respectively. Incubation was continued for another 3hours for complete deactivation of unbound genetic materials. This whole process was monitored by optical density $OD_{600}$ measurement, physical observation through light microscopy and determination of CFU for viable cell count through platting dilutions of culture medium on LB agar plates and incubating them ON at 36°C.

### 2.2.9 Determination of Viable cell in culture medium

Standard automated plating procedure was carried out for determination of CFU in culture medium used for BG production. During this procedure 50µl or 100µl serial dilutions using 0.9% NaCl solution or pure culture medium was subjected to plain LB plates through special spiral platter (WSAP system; DON Whitley Scientific Limited, West York Shire, UK). The LB plates were grown at 36°C over night in Incubators. The next day the colonies were counted using colony counter machine 3.15 (Synoptic Ltd., Cambridge, UK) using the program Synbiosis ProtoCOL.

### 2.2.10 Quantification of plasmid DNA by real time quantitative PCR (qPCR)

Approximately 1ml of sample was taken at different time points to analyze the amount of plasmid DNA retained inside the BGs. The pellets were treated for plasmid DNA isolation as described in (section 2.2.3). Real time quantitative PCR (qPCR) was performed for quantification of mcDNA, mpDNA and mopDNA. In iQ 96 well transparent PCR plates (Bio-Rad) containing 12.5ml of iQ-SYBR Green super mix (Bio-Rad), 1µM of each primer (Microsynth AG), 5µl of template DNA (see isolation of plasmid DNA) in a final volume of 25µl. The PCR plates were sealed using Microseal 'B' (Bio-Rad) adhesive based sealing to prevent evaporation of sample. Following primers listed in the (Table.1) below were designed using primer3 web based tool [77] and were used for the quantification of mcDNA, mpDNA and mopDNA where as for quantification of gentamycin resistance cassette the primers designed by Firas Allaham was used [78].

For generating real time PCR data MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guideline was followed [79]. BioRad iCycler iQ

multiple-color real time PCR detection system with following thermal cycling condition of 95°C for 3min, followed by 30 cycles of 94°C for 40sec and 60°C for 60sec was used to amplify the desired regions. Fluorescence of SYBR Green dye was measured during each extension step. To check the specificity of product amplified the sample was subjected to a final step of melting gradient form 60°C to 95°C with interval of 10sec. a 10 fold serial dilution of minicircle, miniplasmid, or mother plasmid was used to perform standard curve for DNA quantification. All samples and standards were quantified in duplicates and the data analysis was carried out using special software provided by the manufacturer.

**Table.1. List of primers used for real time PCR quantification**

| Template | Amplified region | Primer name and primer sequence | Fragment size and Ref |
|---|---|---|---|
| Purified mcEYFP | 1939-1960nt 7805-7825nt | McXF: 5'-GTGGTTTGTCCAAACTCATCAA-3' McSR: 5'-ACATGAGCAGATCCTCTACGC-3' | 221bp* |
| Purified mpDNA | 7572-7591nt Ori 2124-2143nt ParA | MpAF: 5'-TTTGCAAGCAGCAGATTACG-3' MpYR: 5'-CGCAGCAGCAAAAATAAAAG-3' | 238bp* |
| mopDNA | 1939-1960nt 2124-2143nt ParA | McXF: 5'-GTG GTT TGT CCA AAC TCA TCA A-3' MpYR: 5'-CGC AGC AGC AAA AAT AAA AG-3' | 205bp* |
| pGLNIc | Gentamycin resistance gene | Lys-Gent-RT-Fwd: 5'-CGATGTTACGCAGCAGGGCAG-3' Lys-Gent-RT-Rev: 5'-CGATGAATGTCTTACTACGGAG-3' | 194bp [78] |

* (This work) Primers designed using web based primer3 software using default settings; mcEYFP, minicircle encoding enhanced yellow fluorescent protein; mpDNA, mini plasmid DNA; mopDNA, mother plasmid DNA;

## 2.3 Results

### 2.3.1 Production of mcDNA and mpDNA by site-specific ParA recombination

For standard curve analysis in qPCR purified mcDNA and mpDNA is critical. The ParA resolvase gene in plasmid p4aEYFP-C1 (Fig.4.a) is kept under the expressional control of pBAD promoter. Bacterial strain *E. coli* C2988J and *E. coli* MC4100 carrying plasmid p4aEYFP-C1 (Fig.4.a) and plasmid p3aEYFP-C1 (Fig.4b) were used in this experiment to check the recombination pattern of both plasmids in different bacterial strains. The pBAD promoter was induced by the addition of 0.25% L-(+)-arabinose at time point 0 min, which

results in ParA mediated recombination of mother plasmid into mpDNA and mcDNA in plasmid p4aEYFP-C1 (Fig.5a), whereas no recombination is seen in plasmid p3aEYFP-C1 (Fig.5b) due to absence of *res1* site. Partial recombination seen in bacterial strain *E. coli* C2988J carrying plasmid p4aEYFP-C1 (Fig.5a. lane 5, 6 and 7) as compared to the recombination event documented in *E. coli* MC4100 carrying the same plasmid (Fig.5c).

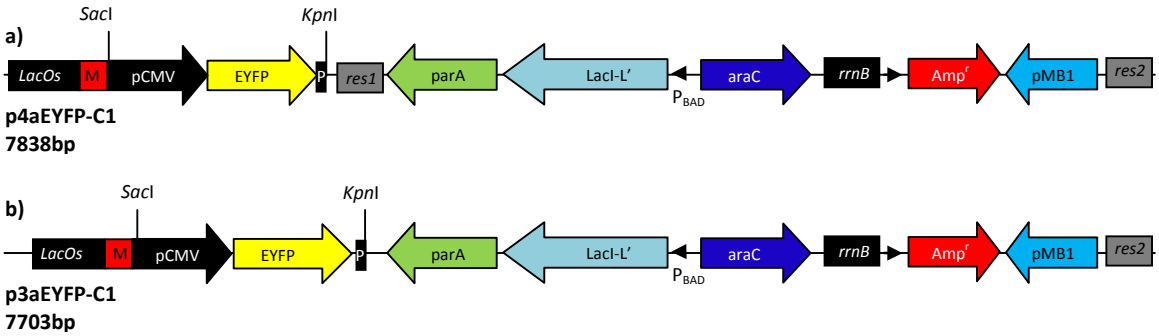


**Fig.4.** ***a) Map of plasmid p4aEYFP-C1; b) Map of plasmid p3aEYFP-C1;*** *(for detail of plasmid p4aEYFP-C1 please see Fig.3b. and for detail of plasmid p3aEYFP-C1 please see Fig.3c respectively)*

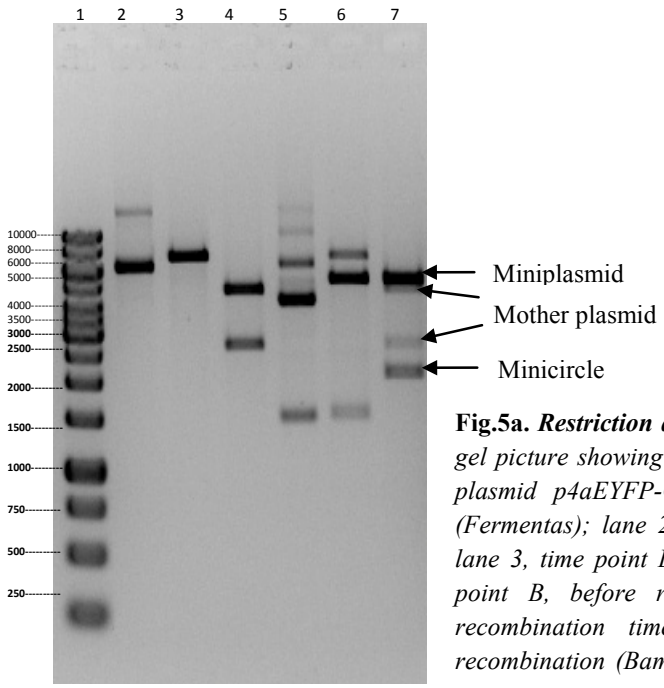


**Fig.5a.** ***Restriction analysis of recombination product;*** *1% agarose gel picture showing recombination event in E.coli C2988J carrying plasmid p4aEYFP-C1 ; lane 1, GeneRuler™ 1kb DNA ladder (Fermentas); lane 2, time point B, before recombination (un-cut); lane 3, time point B before recombination (BamHI); lane 4, time point B, before recombination (NsiI); lane 5, 60min after recombination time point E (un-cut); lane 6, 60min after recombination (BamHI); lane 7, 60 min after recombination (NsiI)*

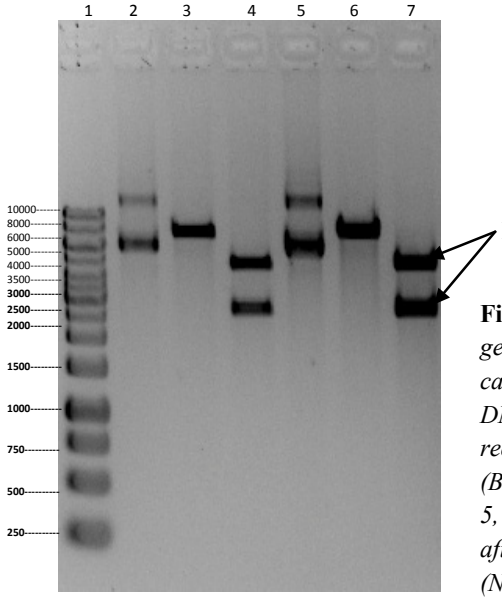


**Fig.5b.** ***restriction analysis of recombination product;*** *1% agarose gel picture showing no recombination activity in E.coli C2988J carrying control plasmid p3aEYFP-C1; lane 1, GeneRuler$^{TM}$ 1kb DNA ladder (Fermentas); lane 2, time point B , before recombination (un-cut); lane 3, time point B , before recombination (BamHI); lane 4, time point B, before recombination (NsiI); lane 5, 60min after recombination time point E (un-cut); lane 6, 60min after recombination (BamHI); lane 7, 60 min after recombination (NsiI) no recombination is observed in control plasmid.*

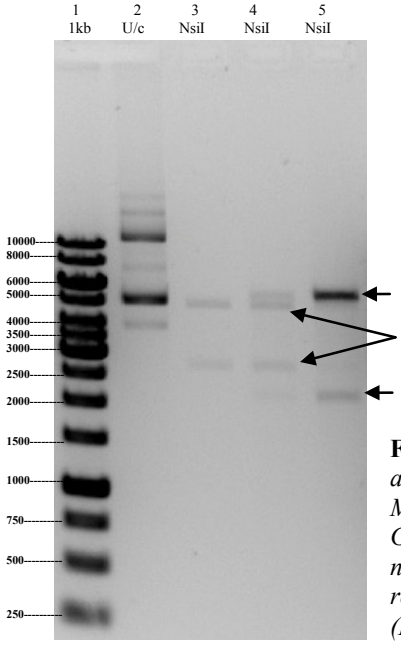


**Fig.5c.** ***Restriction analysis of recombination product;*** *1% agarose gel picture showing recombination event in E.coli MC4100 harbouring plsmid plasmid p4aEYFP-C1; lane 1, GeneRuler$^{TM}$ 1kb DNA ladder(Fermentas); lane 2, uncut over night culture of p4aEYFP-C1; lane 3, timepoint B before recombination (NsiI); lane 4, 30 min after recombination (NsiI); lane 5, 60 min after recombination (NsiI)*

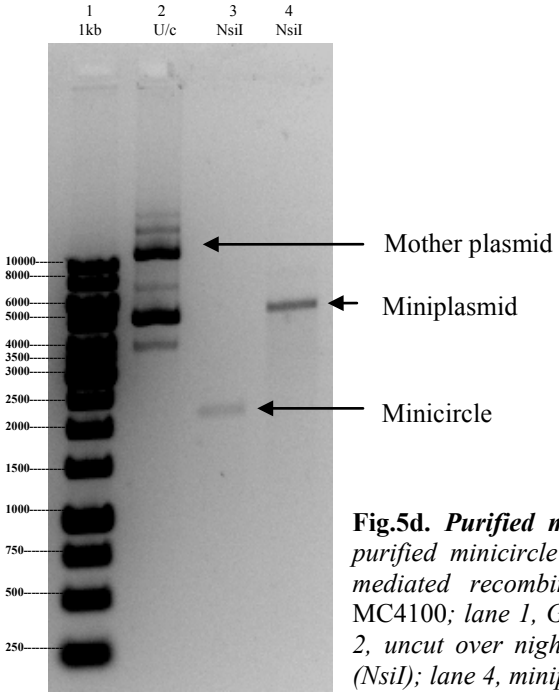


**Fig.5d.** ***Purified mcDNA/mpDNA*****;** *1% agarose gel picture of purified minicircle and miniplasmid DNA obtained from ParA mediated recombination of plasmid p4aEYFP-C1 in E.coli.* MC4100*; lane 1, GeneRuler*$^{TM}$ *1kb DNA ladder(Fermentas); lane 2, uncut over night culture of p4aEYFP-C1; lane 3, minicircle (NsiI); lane 4, miniplasmid (NsiI)*

No recombination event was seen before the induction of ParA resolvase gene in both of the bacterial strains (Fig.5a. lane 2, 3 and 4) and (Fig.5c. lane 3). The negative control lacking one resolution site does not show any recombination pattern before and after the addition of L-(+)-arabinose (Fig.5b). The recombination of plasmid p4aEYFP-C1 in *E. coli* MC4100 was continued for 60 min and the cells were harvested for mcDNA and mpDNA isolation to be used for real time PCR standards. Clear bands of purified mcDNA and mpDNA can be seen in lane 3 and 4 at desired band positions respectively (Fig.5d).

### 2.3.2. Western blot analysis for expression of LacI-L'

Plasmid p4aEYFP-C1 is newly constructed and the (LacI-L' / ParA) expression cassette is inverted therefore it is necessary to do expression study for expression of cloned fusion protein LacI-L' through western blot analysis. The LacI-L' protein in plasmid p4aEYFP-C1 (Fig.4a) and p3aEYFP-C1 (Fig.4b) is under the expressional control of pBAD promoter which is induced by the addition of 0.25% L-(+)-arabinose. To determine the expression of LacI-L' fusion protein after the addition of L-(+)-arabinose, 1ml samples were collected before, in the mid and at the end of protein expression. The expressed fusion protein

was detected through anti Lac repressor serum. The clear background expression of LacI-L' can be seen at about ~45 kDa at the beginning of time point C 20 min after induction (Fig.6a), which is giving stronger signals at time point D and E (Fig.6b), which corresponds to 40 and 60 min post induction respectively. The clear bands at desired ~45 kDa correlates to the fact that LacI-L' was expressed by the addition of L-(+)-arabinose which was added at time point B. No expression of LacI-L' fusion protein observed at time point B which is 0 min and the time point of arabinose addition (Fig.6a).

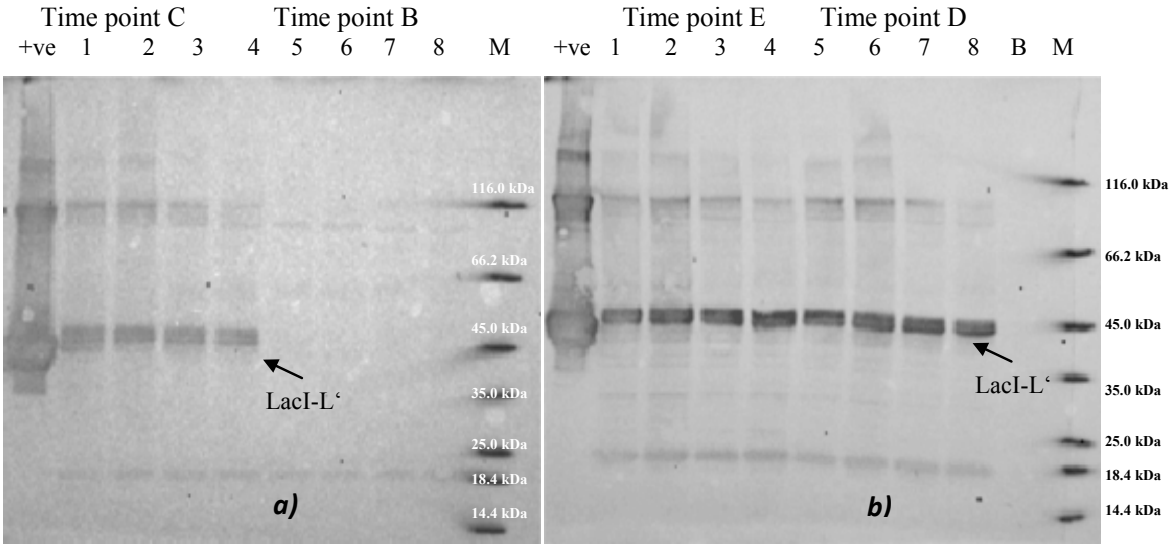


**Fig.6.** ***Western blots analysis for LacI-L';*** *Western blots analysis for detection of fusion protein LacI-L' with anti LacI antiserum in E. coli C2988J harboring plasmid p4aEYFP-C1(lane 3,4,7 and 8) and p3aEYFP-C1(lane 1,2,5,6), LacI-L' fusion protein is under control of pBAD promoter which is induced by the addition of 0.25% L-(+)-arabinose at time point B;* ***a)*** *Clear band of LacI-L' fusion protein can be seen around ~45 kDa at time point C, 20 min after L-(+)-arabinose induction (lane 1, 2, 3and 4) no band detected at the given range at time point B, 0 min time of induction (lane 5, 6, 7 and 8) (+ve)= positive control; M, = unstained protein molecular weight marker (Fermentas);* ***b)*** *clear band of LacI-L' seen around time point D(lane 1,2, 3 and 4) and E (lane 5, 6, 7 and 8) lane 9, Blank; M= unstained protein molecular weight marker (Fermentas); and (+ve) is the positive control.*

### 2.3.3 Transfection efficiency of plasmid p4aEYFP-C1 and p3aEYFP-C1

To evaluate the expression of newly cloned gene encoding Enhanced Yellow Fluorescent Protein (EYFP) which is kept under expression of cytomegalovirus intermediate early (CMV-IE) promoter in plasmid p4aEYFP-C1 and p3aEYFP-C1 (Fig.4. a and b) the CCL-20.2 cells were transfected using TurboFect$^{TM}$ and plasmid DNA from both constructs

i.e. p4aEYFP-C1 and p3aEYFP-C1. This has to be done before the generation of BG loaded with mcDNA carrying the reporte gene under the CMV-IE promoter. The CCL-20.2 cells incubated with plasmid p4aEYFP-C1 alone for 24 and 48 hours showed no fluorescence when observed under light fluorescent microscope (Fig.7.a). The CCl-20.2 cells transfected with 1µg DNA of commercial plasmid pEYFP-C1 using TurboFect$^{TM}$ showed weaker signals (Fig.7.b) as compared to both of the newly constructed plasmids i.e. p4aEYFP-C1 and p3aEYFP-C1 using same amount of DNA. The highest fluorescent signals were recorded in cells transfected with plasmid p4aEYFP-C1 where as medium (Fig.7.d) compared to this, medium fluorescence signals were recorded in cells transfected with plasmid p3aEYFP-C1 (Fig.7c) suggesting the better efficiency of cloned genes in SIP plasmids. This might be due to the removal of DNA sequences in between the EYFP protein and polyA tail during the construction of plasmid pEYFP-C1-MCS (Fig.3a) which is used further to clone plasmid p4aEYFP-C1 and p3aEYFP-C1 (Fig 3b. and 3c. respectively).

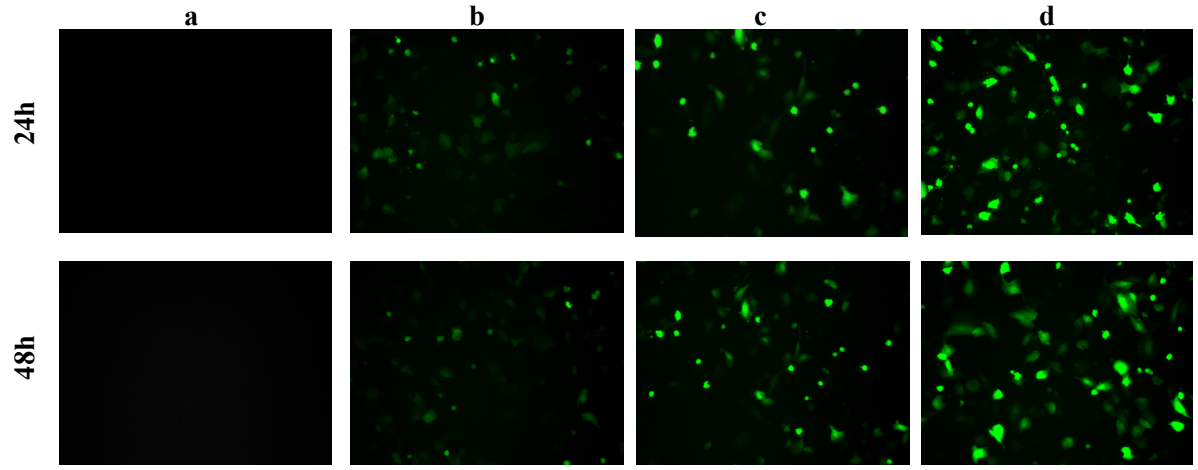


**Fig.7.** ***Transfection of CCL-20.2 cells;*** *Expression of enhanced yellow fluorescent protein EYFP in CCl-20.2 cell line 24 hours and 48 hours post transfection.* ***a)*** *Ctrl; Control, 4x10$^{4}$ cells plus naked DNA;* ***b)*** *pEYFP-C1 plus TurboFect$^{TM}$;* ***c)*** *p3aEYFP-C1plus TurboFect$^{TM}$;* ***d)*** *p4aEYFP-C1plus TurboFect$^{TM}$*

Preliminary results of cells transfected with BGs carrying enhanced yellow fluorescent protein minicircles (mcEYFP) produced by recombination of plasmid p4aEYFP-C1 gave weak expression signals in macrophages RAW-264.7 cells (Fig.8). The experiment has to be repeated after production of BGs harboring mcDNA on large scale under controlled environment. After which conclusions could be drawn for use of BG loaded with mcDNA as a therapeutic target.

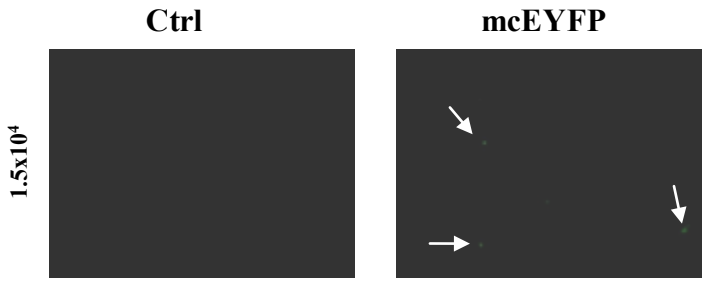


**Fig.8.** ***Transfection of RAW-264.7 cells with BGs carrying mcDNA;*** *BGs carrying mcDNA encoding enhanced yellow fluorescent protein EYFP resulted in weak expression of clone gene in RAW-264.7celsl line 48 hours post transfection. Ctrl, Control $1.5_X10^4$ cells plus naked DNA; mcEYFP, in vivo loaded Bacterial Ghosts (BGs) with minicircle encoding enhanced yellow fluorescent protein (mcEYFP); no signal seen in control.*

### 2.3.4 Production of mcDNA loaded BGs devoid of mpDNA and un-recombined mopDNA

For producing BGs harboring mcDNA that are free of mpDNA and un-recombined mopDNA, the newly constructed plasmid p4aEYFP-C1 (Fig.3b) was co-transformed with plasmid pGLNIc (carrying lysis and SNUC gene Fig.9) in *E. coli* Nissle 1917.

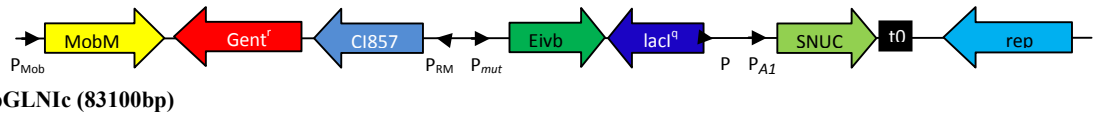


**Fig.9.** ***Map of plasmid pGLNIc;*** *$P_{Mob}$, promoter of mob gene; MobM, mutated mobilization sequence; Gent$^r$, gentamycin resistance cassette; CI857, thermosensitive allele of the λ phage repressor gene; $P_{RM}$ / $P_{mut}$ , mutated promoter of λ phage; Eivb, in vivo biotinylated lysis protein E sequence of bacteriophage phiX174; SNUC, Staphylococcus aureus nuclease A; t0, terminator sequence; rep, origin of replication (adapted and modified from PhD thesis of Timo Lanngamann 2011)*

The plasmid p3aEYFP-C1 (Fig.3c) was used as a control plasmid due to absence of *res1* site hence it does not recombine. Expression of LacI-L' / ParA recombinase protein (which is controlled by pBAD promoter) was induced at an optical density $OD_{600}$ of about 0.2-0.3. by addition of 0.25% L-(+)-arabinose which results in recombination of mopDNA into mpDNA and mcDNA and synthesis of membrane anchored fusion protein LacI-L'. Drop in CFU was noted in samples upon addition of L-(+)-arabinose (Fig.10. time point -60 min) which kept on dropping after the addition of 5mM IPTG (Fig.10 time point -30 min) indicating an inhibitory effect of chemical manipulation on cell viability in case of above mentioned plasmid combinations. No inhibitory effect was observed in untreated control

group where the $OD_{600}$ and CFU values did not change (Fig■10and □ respectively). Obtained results from three independent experiments showed 98.62% drop in CFU count in samples with ParA and *gene E* induction (Fig.11, empty bar). The same plasmid combination when treated with recombination lysis and SNUC (RLS) showed 99.94% bacterial inactivation (Fig.11, striped bar). The highest bacterial inactivation was recorded in nose flask with induced gene *E* followed by nuclease inactivation close to 99.97% (Fig.11 dark bar).

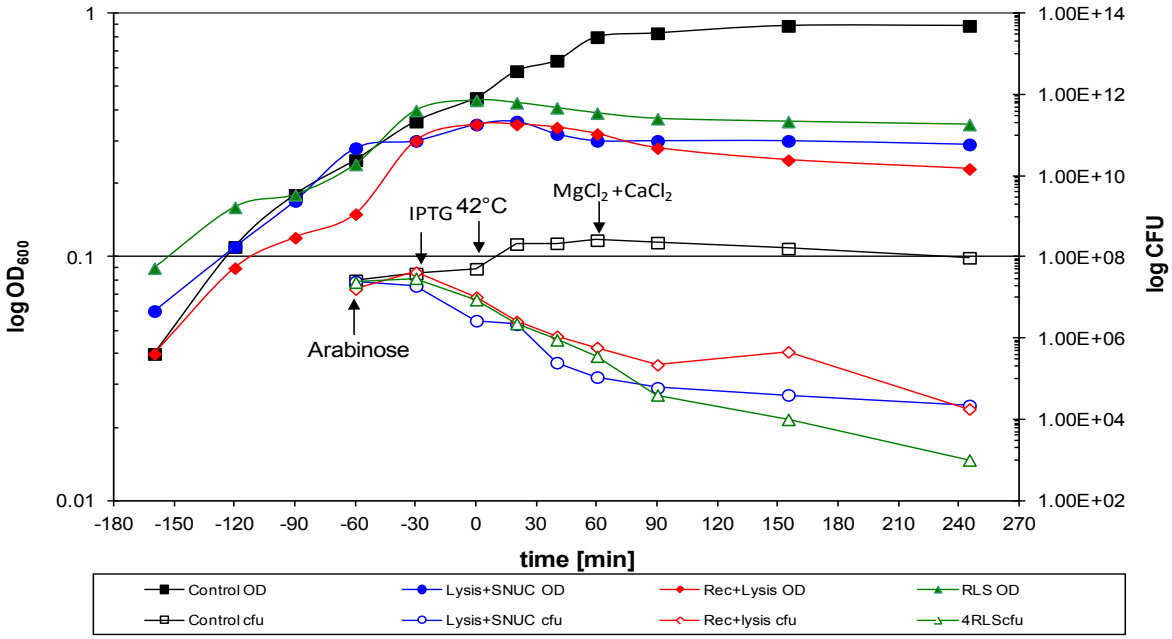


***Fig.10. OD and CFU count of RLS test;*** *E. coli Nissle 1917 carrying plasmid p4aEYFP-C1+pGLNIc (Red and Green) ; p3aEYFP-C1+pGLNIc (Black and Blue); Drop in CFU is recorded in samples treated with L-(+)-arabinose (0.25%) at time point -60 min. no significant change in OD and CFU values were observed in untreated control(black line ■ / □ respectively)*

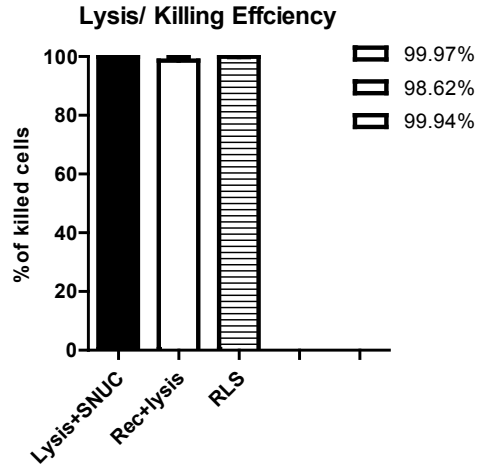


**Fig.11.** ***Percentage of killed or inactivated bacterial cells obtained through CFU count;*** *99.97% inactivation of bacterial cells in samples induced with lysis gene E and SNUC activation (dark bars); 98.62% reduction in CFU count in samples treated with arabinose and temperature up shift of 42° (empty bars); and 99.94% decrease in CFU of samples with ParA recombination followed by induction of gene E and finally SNUC activation RLS (recombination lysis and SNUC) (horizontal stripe bars) (mean SD of 3 different experiments)*

The qPCR analysis for detection of lysis plasmid with primers designed for gentamycin resistant cassette (Table.1) is an important tool for monitoring the change in amount of genetic material during growth, lysis and SNUC activation. The plasmid DNA samples from the above experiments were subjected to real time PCR for monitoring the gentamycin resistance gene present on the plasmid pGLNIc. Loss in gentamycin resistant gene was recorded to be 62.57% in the samples treated with arabinose and lysis activity (Fig.12 white bar). The highest inactivation of genetic material was recorded in samples with induced gene *E* and SNUC activation which was close to 99.22% (Fig.12, black bar). Compared to this the samples which were treated with 0.25% L-(+)-arabinose before the induction of gene *E* and activation of SNUC through addition of $CaCl_2$ and $MgCl_2$ (0.01M and 0.001M respectively) showed slight drop in genetic inactivation 96.85% (Fig.12, strip bar). Form this it can be concluded that the recombination event play an important role in decreasing the enzymatic activity of SNUC.

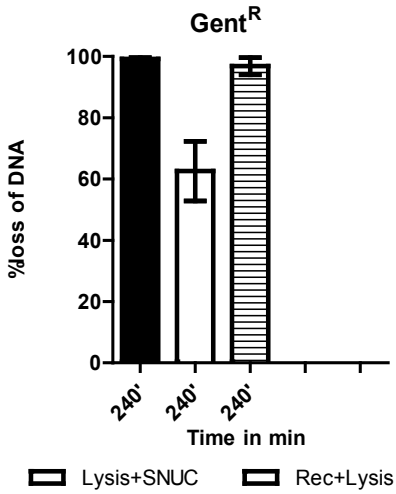


***Fig.12. Percentage loss of lysis plasmid at the end of chemical and or physical induction;.*** *99.22% reduction in gentamycin resistant cassette in samples with induced gene E and SNUC activation (dark bar); 62.57% reduction of lysis plasmid in samples with ParA recombinase and lysis gene E induction (empty bar); and 96.85% decrease in genetic material in samples with ParA recombination followed by induction of gene E and finally SNUC activation (horizontal stripe bar) (mean of 3 individual experiments are indicated by error bars)*

### 2.3.4.1 Effect of L-(+)-arabinose on combination of p4aEYFp-C1 and pGLNIc

To investigate whether the final lysis and inactivation of the bacterial culture is not due to L-(+)-arabinose addition but due to induction of lysis gene *E* and SNUC activation it was necessary to check the effect of L-(+)-arabinose alone on bacterial cells carrying both plasmids i.e. p4aEYFP-C1(Fig.3b) and pGLNIc (Fig.9). To investigate further the effect of L-(+)-arabinose on each of the plasmids separately the *E. coli* Nissle 1917 carrying plasmid p4aEYFP and plasmid pGLNIc were also included in the above experiment. Drop in CFU was recorded for the first 2 hours upon addition of L-(+)-arabinose in bacteria carrying both

the plasmids i.e. (p4aEYFP-C1 and pGLNIc) followed by stabilization of bacterial cells and normal growth was observed at later stages (Fig.13 ○ blue line). No change in $OD_{600}$ and CFU count was recorded in cells carrying plasmid pGLNIc or p4aEYFP-C1 separately upon addition of L-(+)-arabinose (Fig.13 □ black and Δ red). This impaired growth of cells upon L-(+)-arabinose addition was only seen in *E. coli* Nissle 1917 carrying both the plasmids i.e. p4aEYFP-C1 and pGLNIc. Hence it is concluded that the final inactivation achieved in recombination lysis test or RLS test is due to the effect of BG formation and nuclease activity and not due to the L-(+)-arabinose addition.

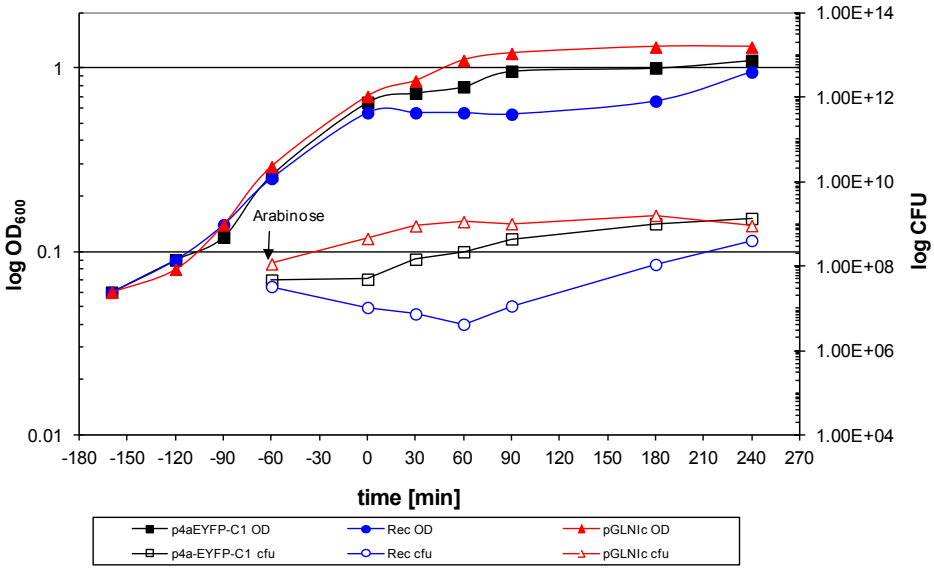


***Fig.13. Effect of L-(+) arabinose;*** *OD and CFU count of E. coli Nissle 1917 carrying plasmid p4aEYFP-C1+pGLNIc (Blue line ● ○); p4aEYFP-C1 (Black line ■ □) and pGLNIc(red line▲ Δ); drop in CFU recorded in E. coli Nissle 1917 carrying both the plasmids p4aEYFP-C1+pGLNIc upon addition of 0.25% L-(+)-arabinose for first 2 hours where as cell started to recover and normal growth seen at the end of experiment; no effect on cell growth observed in E. coli Nissle 1917 carrying p4aEYFP-C1 or pGLNIc separately upon addition of 0.25% L-(+)-arabinose*

### 2.3.4.2 Effect of IPTG addition at different time points

It was shown in previous studies that the activity of SNUC is solely dependent on addition of $CaCl_2$ and $MgCl_2$, and is independent of time point of nuclease induction through addition of IPTG [59]. In this part the effect of IPTG addition at different time points was investigated. For this reason *E. coli* Nissle 1917 carrying two plasmids p4aEYFP-C1 and pGLNIc were subjected to 7 different controlled physiological conditions i.e. *E* lysis alone; ParA mediated recombination alone; SNUC activation alone; *E* lysis and SNUC activity; ParA recombination and SNUC activity; ParA recombination and lysis and finally RLS

activity altogether (Fig.14 & 15). Slight modification was made in design of this experiment by decreasing the amount of L-(+)-arabinose to 0.06% (it has been shown in previous studies that the concentration of arabinose can be manipulated without affecting the desired expression Schlacher and Abbas).

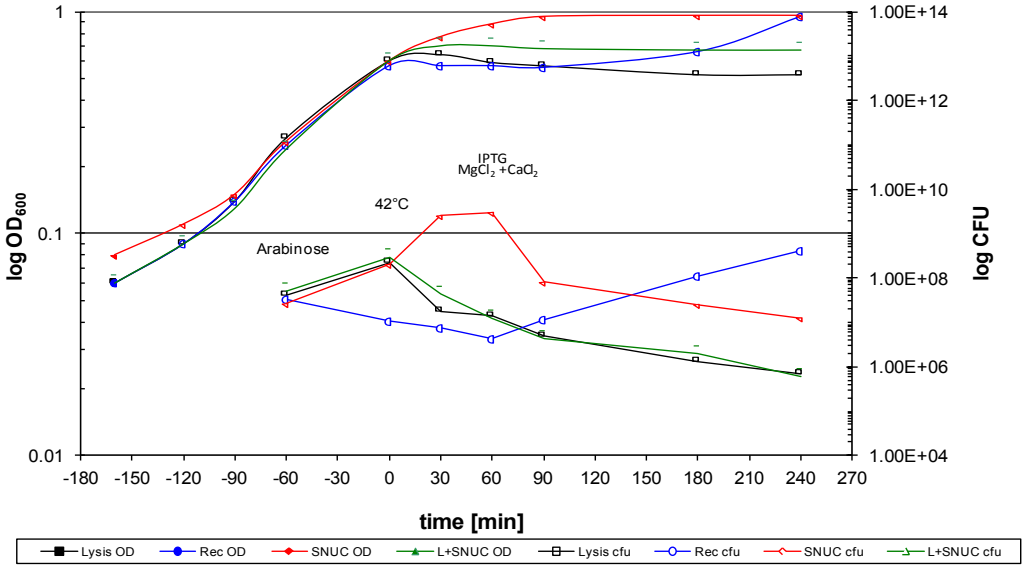


**Fig.14.** ***Effect of IPTG addition on SNUC activity;*** *OD and CFU graph of lysis test of E. coli Nissle 1917 carrying plasmid p4aEYFP-C1 and pGLNIc; drop in CFU seen after addition of L-(+)-arabinose (0.06%) at time point -60 min. (Blue); lysis only (Black); lysis+SNUC (Green); SNUC alone (Red).*

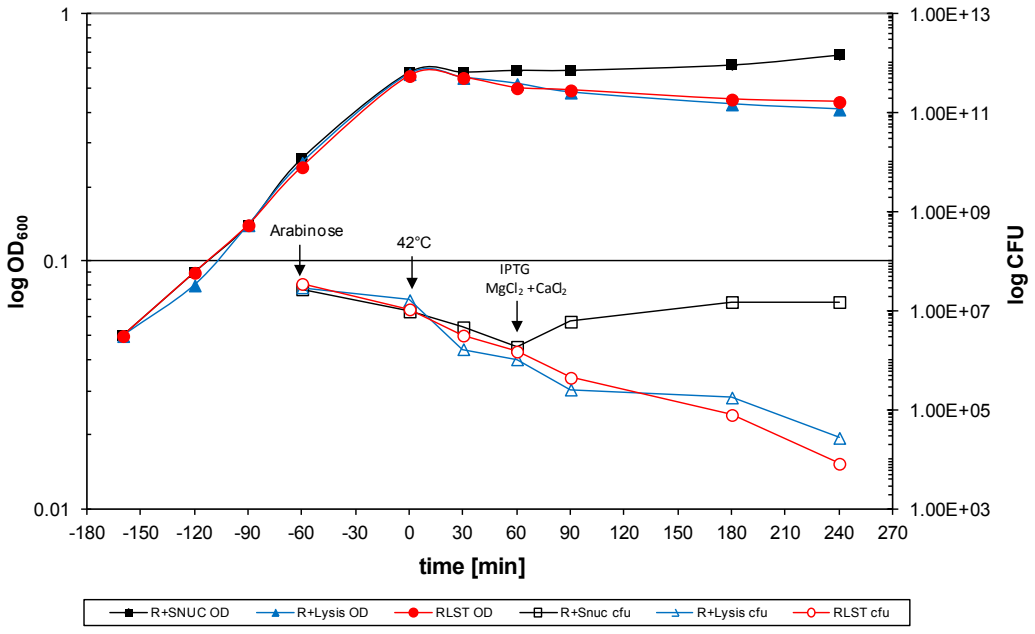


**Fig.15.** ***Effect of IPTG addition on SNUC activity;*** *OD and CFU count of E. coli Nissle 1917 carrying plasmid p4aEYFP-C1 and pGLNIc. Drop in CFU seen after addition of L-(+)-arabinose 0.06% at time point -60 min. recombination+SNUC (black); recombination lysis (blue); recombination+lysis+SNUC (RLST) (red).*

Results obtained from the above experiment showed lysis efficiency of 99.69% in the *E. coli* Nissle carrying plasmid p4aEYFP-C1 and pGLNIc (Fig.14 black line) which is slightly higher than in samples with ParA mediated recombination followed by gene *E* lysis where the 98.62%. of bacterial cells were lysed (Fig.15 blue line) this shows that the cells lysed better in the absence of L-(+)-arabinose this observation was verified through qPCR analysis of DNA samples from above two conditions which showed 75.30% loss in lysis plasmid in group with gene *E* induction alone as compared to 62.57% in samples treated with arabinose and gene *E* induction. It was also found out that the addition of IPTG 30 min prior to *E* lysis is crucial for achieving maximum inactivation of genetic material. Drop in SNUC activity is observed upon simultaneous addition of IPTG and $CaCl_2$ and $MgCl_2$ 60 min after the completion of *E* mediated lysis of bacterial cells. This drop in SNUC activity was supported through qPCR which showed better inactivation of genetic material in sample where IPTG was added -30 min / before the induction of lysis gene *E* than the sample in which IPTG was added 60 min after gene *E* activation. The amount of genetic material hydrolyzed by SNUC where IPTG was added 60 min after gene *E* mediated lysis induction dropped by 15-25% in two different cases (Fig.16).

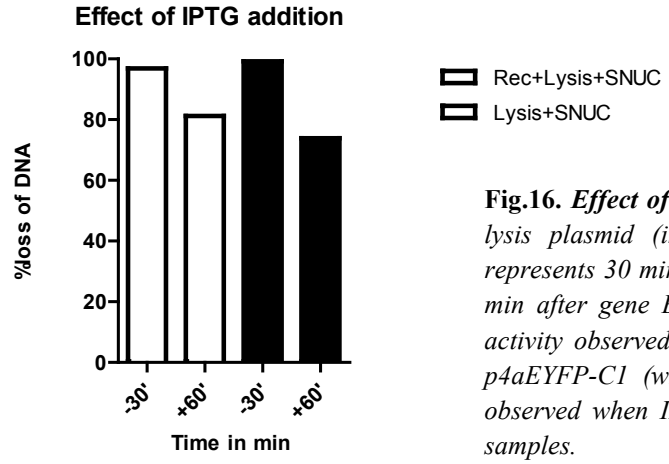


**Fig.16.** ***Effect of IPTG addition on SNUC activity;*** *Decrease in lysis plasmid (in %) was recorded by qPCR analysis. -30 represents 30 min before gene E induction and +60 represent 60 min after gene E induction there is 15% decrease in nuclease activity observed when IPTG was added at +60 in recombined p4aEYFP-C1 (white bars); 25% decrease in nuclease activity observed when IPTG was added at +60 min in un-recombined samples.*

### 2.3.5 Quantification of mcDNA retained inside the BGs

The primary aim of this study was to immobilize the mcDNA inside the BGs and to get rid of the unwanted mpDNA which is a by-product of ParA mediated recombination and un-recombined mopDNA. The ParA recombination of plasmid p4aEYFP-C1 in bacterial strain *E. coli* Nissle 1917 was carried out by addition of L-(+)-arabinose in which the

mcDNA is produced. Two different methods were applied to get rid of unwanted mpDNA which included i) *E* mediated lysis of bacterial cell resulting in expulsion of unbound mpDNA ii) *E* mediated lysis of bacterial cells followed by SNUC activity in order to hydrolyze the available mpDNA and un recombined mopDNA and achieve maximum inactivation of mpDNA in BG pellet. 1ml bacterial culture sample were collected at different time points before and after the recombination; before and after the lysis and before and after the SNUC activation, where ever needed. *In vivo* loading efficiency of BGs with mcDNA was determined by qPCR amplifying the 221bp region of fully formed mcDNA using primers McXF and McSR (for primer sequence see Table.1). The amount of mpDNA retained in the BG pellet was calculated through amplifying the 238bp sequence present on fully formed mpDNA using primer MpAF and MpYR (prime sequence in Table.1). In samples with gene *E* mediated lysis loss in mpDNA was recorded to be only 18.07% (Fig.17 dark bar) however, the percentage loss in amount of mpDNA in samples with induced *gene E* and SNUC activity was considerably high i.e. 99.48% (Fig.17 empty bar).

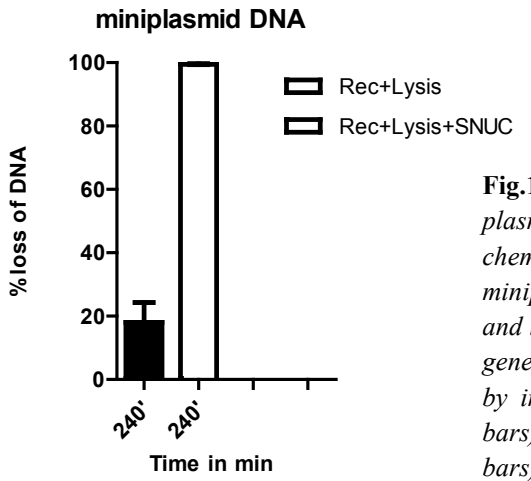


**Fig.17.** ***Quantification of mpDNA;*** *Decrease in amount of mini plasmid DNA in the pellet of samples taken 240 min after the chemical and physical induction. 13.67% reduction in miniplasmid DNA in pellet of samples with parA recombinase and lysis gene E induction (Dark bars); and 99.48% decrease in genetic material in samples with parA recombination followed by induction of gene E and finally SNUC activation (Empty bars)( mean of two individual experiments shown with error bars)*

Compared to this the amount of mcDNA retained inside the BG is recorded to be 68.22% in samples with induced gene *E,* which means 32.88% of mcDNA is lost during the *E* mediated lysis process (Fig.18 dark bar), whereas there was unexpected decrease in amount of mcDNA in sample with induced gene *E* followed by SNUC activation which is close to 97.63% (Fig.18 empty bar). This means only 2.37% of mcDNA is still retained inside the BGs.

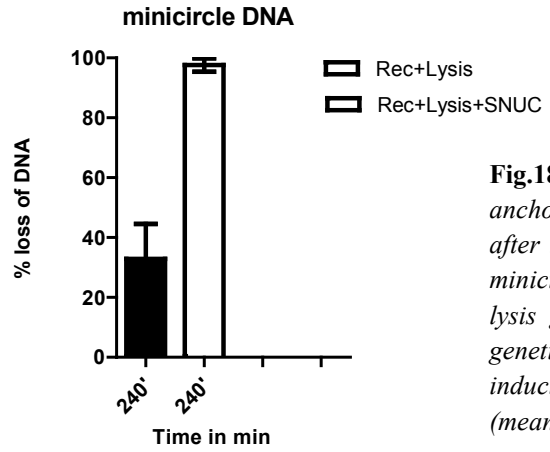


**Fig.18.** ***Quantification of mcDNA;*** *Decrease in amount of anchored minicircle DNA in the pellet of samples taken 240 min after the chemical and physical induction. 32.88% reduction in minicircle DNA in pellet of samples with parA recombinase and lysis gene E induction (Dark bars); and 97.63% decrease in genetic material in samples with parA recombination followed by induction of gene E and finally SNUC activation (Empty bars) (mean of 3 individual experiments are indicated by error bars)*

However by comparing the amount of mcDNA and mpDNA per BG in set of 3 experimets below it is observed that there is a huge drop in mpDNA copy / BG (8.27mpDNA / BG) where samples are treated with SNUC. Howereve it is to be noted that amount of mcDNA did not drop with that ratio as is seen on mpDNA. The amount of mcDNA retained in BGs treated with SNUC was calculated to be 25.7 copies of mcDNA / BG (Table.2).

**Table.2. Amount of mcDNA and mpDNA retained inside the BGs**

| Method | mcDNA ng. / total no of cells | mcDNA copy /BG | mpDNA ng. / total number of cells | mpDNA copy / BG |
|---|---|---|---|---|
| ParA recombination and *E* lysis | 42.96ng / $5.59x10^7$ | 322 | 166ng / $6.49x10^7$ | 412 |
| ParA recombination *E* lysis and SNUC | 2.62ng / $4.28x10^7$ | 25.7 | 1.39ng / $2.71x10^7$ | 8.27 |

Amount of mcDNA and mpDNA calculated through qPCR: mcDNA generated from two different techniques. The plasmid ng is converted into the copy number based on following formulae [copy number = (ng of DNA x 6.022 x $10^{23}$) / (size of plasmid in bp. x 1 $x10^9$x 660)] where $6.02214199x10^{23}$ is Avogadro's number. Size of mcDNA= 2173bp, and mpDNA= 5666bp; *mean of three individual experiments*

## 2.4 Discussion

It is understood that the mcDNA devoid of bacterial sequences is a novel DNA vaccine candidate, although there are limitations in its practical implications due to its laborious production and purification [22, 29-31]. The efficient recombination of plasmid DNA to produce minicircles have been addressed by the introduction of *in vivo* loaded

mcDNA BGs, which showed recombination efficiency of around 100% and yet still needs to be free of mpDNA that are retained in the pellet of BGs for its practical use in clinical trials [57]. Reduction of mpDNA in the BG preparation is the main objective in this study. Combination of mcDNA immobilization inside BGs and reduction of un-wanted mpDNA / mopDNA sequences through hydrolysis activity of SNUC is investigated to achieve final goal. In this investigation 4 different protocols are combined together to get *in vivo* loaded mcDNA free of unwanted mpDNA / mopDNA sequences, including, i) mcDNA production through parA recombination, ii) immobilization of mcDNA inside the Gram-negative bacteria through SIP system, iii) BG production through gene *E* mediated lysis, iv) elimination of un-recombined mopDNA and mpDNA through SNUC.

The result obtained from this study highlights the inhibitory effect of L-(+)-arabinose addition on bacterial cells carrying both plasmids p4aEYFP-C1 and pGLNIc. This inhibitory effect was independent of bacterial strain used for mcDNA production. Three different *E. coli* stains (C2988J, MC4100 and Nissle 1917) carrying plasmid p4aEYFP-C1 and pGLNIc showed similar pattern of CFU drop upon addition of L-(+)-arabinose. However, investigation for revealing the effect of L-(+)-arabinose addition on bacteria carrying these two plasmids individually did not show any adverse effect leading to conclusion that the final drop in CFU is due to the gene *E* mediated lysis and SNUC inactivation of the bacterial cells. Thus the real time PCR results for quantification of anchored mcDNA and unbound retained mpDNA are considered accurate. In this study the extra step of nuclease induction serves as the quality control measure for production of mcDNA loaded BGs. The remaining amount of mpDNA inside the BG preparations can be a serious threat for its use in clinical trials, here in this study it has been shown through qPCR the reduction in mpDNA concentration by 99.48% as compared to previous 70% [57]. However during this inactivation procedure the amount of mcDNA was also reduced to critical 2.38% only, which is slightly more than what was predicted. When converted to plasmid copy / BG it corresponded to approx ~23-59 plasmid copies of mcDNA / BG where as previous studies showed that even 50 plasmid copies / BG is enough to get transfection efficiency of around 82% [81]. BGs with mcDNA generated from ParA recombination of plasmid p4aEYFP-C1 through procedure explained earlier has been tested in cell culture and preliminary data showed weak fluorescence of enhanced yellow fluorescent protein in RAW-264.7 cells (Fig.8). These tissue culture experiments for reporter gene activity has to be repeated after production of BGs carrying

mcDNA that are devoid of mpDNA using method developed her in on large scale under controlled conditions before drawing any final conclusion for use of BGs loaded with mcDNA as a therapeutic target.

Another aspect that was studied here was the effect of IPTG addition at different time points on SNUC activity earlier reports suggested that the IPTG addition is independent of time point of its addition and its activation through addition of $CaCl_2$ and $MgCl_2$ however in this study experiments showed that there is a significant drop in killing and hydrolyzing activity of SNUC upon simultaneous addition of IPTG and $CaCl_2$ and $MgCl_2$ (Fig.16). This might be due to the fact that the bacterial cells are already dead or lysed due to addition of L-(+) arabinose and or lysis gene *E* respectively, hence there is not enough bacterial machinery for producing the nuclease enzyme which is crucial for DNA hydrolysis. Thus it is important to induce SNUC gene 30 min before the induction of lysis *gene E* in order to achieve maximum inactivation of mpDNA / mopDNA in prepared BGs.

## 2.5 References


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Chapter III

# Production of minicircle DNA loaded Bacterial Ghosts carrying a reporter gene and its quantification using quantitative Real Time PCR (qPCR)

**Abstract**

Bacterial Ghosts (BGs) loaded with minicircle DNA (mcDNA) carrying reporter genes are important tool for basic research and screening of delivered antigen. In this study the mcDNA harboring enhanced green-yellow fluorescent protein (EYFP), Red fluorescent protein (mCherry), and green fluorescent protein (VenusA206K) was produced along with the expression of lysis gene *E* to get in-vivo loaded mcDNA BGs. The expression of the eukaryotic regulated reporter gene cloned in advanced version of Self Immobilizing Plasmid (SIP) resulted in better fluorescent signals when tested in CCL-20.2 cells. The mcDNA is produced by parA site specific recombination of mother plasmid DNA (mopDNA) and is anchored inside the BGs through specific interaction between membrane anchored LacI-L' fusion protein and the *lac* operator sequence (lacOs) present on the mcDNA followed by gene *E* lysis transformed together on a separate plasmid. Previously the amount of mcDNA anchored inside the BGs was quantified through quantitative real-time PCR (qPCR) by amplification of lacOs site present on mcDNA, whereas miniplasmid DNA (mpDNA) was quantified by amplifying the resistance cassette used for its production. This technique had its limitations as the sequences being used for their quantification is also present on mopDNA, hence these primers could attach and detect the desired sequence independent of their presence on fully formed mcDNA/mpDNA or on un-recombined mopDNA. In this study a new strategy is developed to quantify the amount of mcDNA and mpDNA depending on their subsequent sequences present on recombined (mcDNA/mpDNA) or on un-recombined mopDNA through qPCR. This technique is very simple and highly structured as the final step of melt curve analysis will then verifies the source of quantified mcDNA/mpDNA i.e. from fully formed mcDNA/mpDNA or from un-recombined mopDNA.

## 3.1 Introduction

The use of plasmid DNA as a vaccine has been investigated in most of the recent studies [1-5]. However its practical use is limited due to the absence of efficient delivery system. Among currently available viral and non viral DNA delivery system the non viral delivery system has an edge due to its safety profile, ease of manufacturing and for their better capacity in delivering antigens [6-11]. The presence of unmethylated CpG sequences and antibiotic resistance gene has limited the use of DNA vaccines. For this reason a new smaller size DNA molecule lacking these sequences has been developed which is known as mcDNA [12-14]. These mcDNA not only minimized the risk for the spread of antibiotic resistance among wild type strains but increase the transgene signals in mammalian cells due to their reduced size and absence of unmethylated CpG motifs [13-15]. Minicircles are produced in a different ways through site-specific recombination activity of bacteriophage λ integrase [12, 13, 15], ParA resolvase [16], *Cre* recombinase of bacteriophage P1 [17] and by the ϕC31 integrase [14] in most of *E.coli* bacterial strains. The enzyme listed above recognizes and cleaves the DNA sequences located in between the two corresponding recombination (*rec*) sites. This property of recombinase is used to produce minicircles thus by positioning the origin of replication and the resistance cassette in between these two *rec* sites the mopDNA can be divided into, a replicative mpDNA carrying unwanted bacterial backbone (BB) sequences and the mcDNA carrying only the therapeutic active expression cassette [12-15, 18]. Formally mcDNA was purified through cesium chloride gradient ultra centrifugation which is quite laborious [19] and the recombination process itself was not efficient enough e.g. Darquet *et.al.,* 50%, Kreiss *et.al.,* 85% and chen *et.al.,* 97% [12-15] until the introduction of parA recombination system which was 100% effective and can be achieved in only 30 min [20].

Apart from the safety profile of DNA vaccines, the efficient delivery system is the main focus in designing novel drugs. Bacterial ghost system represents a novel system in delivering the foreign antigens inside the host. BGs due to their intact surface structures act as an adjuvant and can elicit effective humoral and cellular immune response [21]. These BGs are produced by the controlled expression of lysis gene *E* of Phage ϕX174 in Gram-negative bacterium [22, 23]. The gene *E* is kept under the tight control of temperature sensitive $\lambda pR_{mut}$/*CI*857 promoter/repressor system and can be induced by temperature shift of 36°C to 42°C [24]. The expression of lethal gene *E* results in the formation of transmembrane tunnel like structure through which the cytoplasmic contents are expelled in the surrounding medium

due to difference in their osmotic pressure [25-27]. This system together with self immobilizing plasmid is used to produce the self loaded DNA vaccines. In SIP system the plasmid carries the lac operator sequences (lacOs) which is recognized by the LacI-L' anchor protein which is composed of lactose repressor (LacI) and hydrophobic membrane anchor L' derived from phage MS2 [18]. In later study the parA system flanking the antibiotic resistance cassette and origin of replication was combined together with the LacI-L' membrane anchoring and E lysis process to produce in-vivo loaded mcDNA BGs [20]. This process simplified the production of mcDNA by vanquishing the need of laborious purification steps in its preparation.

Real-time quantitative polymerase chain reaction (qPCR) is the method of choice and most commonly used molecular biology technique for detection and quantification of nucleic acid [28]. Its practical implication ranges from techniques used in molecular diagnostics [29] to forensic science [30] and in a wide range of basic research in field of biotechnology. The main advantage of this technique is the elimination of need to amplify and detect the final product separately on agarose gel. This technique analyzes the quantitative relationship between the amounts of target used at the start of reaction to the amount of amplified PCR product at a certain cycle. Previously the qPCR was used to quantify the amount of mcDNA and mpDNA in the BG preparations. Through amplifying the lacOs site present on mcDNA and antibiotic resistance gene present on mpDNA [20]. However there are some limitations in quantification through this method due to presence of above sequences on un-recombined mopDNA which can interfere with the final results. For this reason the final recombination product is subjected to restriction digestion with enzymes cutting once in both mcDNA and mpDNA and through densitometric analysis the recombination efficiency was calculated [31] after which it was decided that the amount of mcDNA / mpDNA being quantified is coming from fully formed mcDNA/mpDNA or from the un-recombined mopDNA. To overcome this problem a new strategy to quantify the amount of mcDNA/mpDNA depending on the presence of their relevant sequence on fully formed mcDNA/mpDNA or on un-recombined mopDNA is presented. These primers presented herein has the ability to generate different size PCR products depending on the form of mopDNA used i.e. recombined or un-recombined and through melt curve analysis at the end of qPCR one can trace the source of quantified product i.e. from fully formed mcDNA/mpDNA or from un-recombined mopDNA. In melt curve analysis a single narrow peak is the indication of a single pure PCR product. This can be

double checked through visual analysis of final qPCR product on 2% agarose gel which will then serve as a quality control criterion in the end.

## 3.2 Material and methods

### 3.2.1 Bacterial strains, growth conditions and plasmids used

Commercially available bacterial strain *E.coli K12* MC4100 [32] *F- Δ(arg-lac)U169 araD139 rpsL 150 ptsF25 fibB5301 rbsR deoC relA1.* from (New England Biolabs) was grown on LB Luria-Bertani (LB) medium [33] when necessary the LB (purchased form Roth) was supplemented with respective amounts of ampicillin and gentamycin at concentration of 100µg/ml and 20µg/ml respectively (Sigma-Aldrich). For pBAD derived plasmid vectors 2% glucose was added for tight repression of arabinose promoter. The parA resolvase was induced by the addition of 0.25% L-(+)-arabinose (Sigma-Aldrich) at optical density 600 of ($OD_{600nm}$ ~0.2 to 0.3). The lysis gene *E* in plasmid pGLysivb [34] was induced with the temperature change from 36°C to 42°C. The Growth and lysis of the bacterial population was monitored by measuring the $OD_{600nm}$, physical observation through light microscopy and the inactivation of cells due to lysis gene *E* was recorded by plating dilutions of bacterial cultures at different time points for colony forming unit count (CFU) as described earlier [35]. Following plasmids has been used in this study, Plasmid pSIPHCNparA [20], plasmid pGLysivb [34], pEYFP-C1 a kind gift by Dr. Monika Sramkova NIH America (Clontech, BD-Biosciences), plasmid pSIPHCNparA-res1 (this work), p2a (this work), p3a (this work), p4a (this work), p4aEYFP-C1 (this work) p3aEYFP-C1 (this work), p4amCherry-C1 (this work), p3amCherry-C1 (this work), p4aVenusA206K-C1(this work) and p3aVenusA206K-C1(this work).

### 3.2.2 Plasmid construction

#### 3.2.2.1 Construction of plasmid p3a

To construct Plasmid p3a two intermediate plasmid constructions (pSIPHCNparA-res1, and p2a) was carried out. For easy handling, plasmid pSIPHCNparA [20] (Fig.1a) was digested with *HindIII* (Fermentas) double cut and was re-ligated to remove the resolution site 1 (res1 140bp). The resulting plasmid was named pSIPHCNparA-res1 (6268bp) (Fig.1a) lacking recombination activity. Suitable restriction sites or multiple cloning sites MCS was

introduced into this plasmid pSIPHCNparA-res1 by digesting it with *Nsi*I (Fermentas) and the 60bp MCS (Synthesized) was inserted into this site the resulting annealed plasmid is then called p2a (6128bp) (Fig.1b). This was done by mixing and incubating two primers synthesized for MCS (MCS1 and MCS2) and by introducing this annealed MCS into respective restriction site present on plasmid pSIPHCNparA-res1 using T4 DNA ligase (New England BioLabs, Germany).

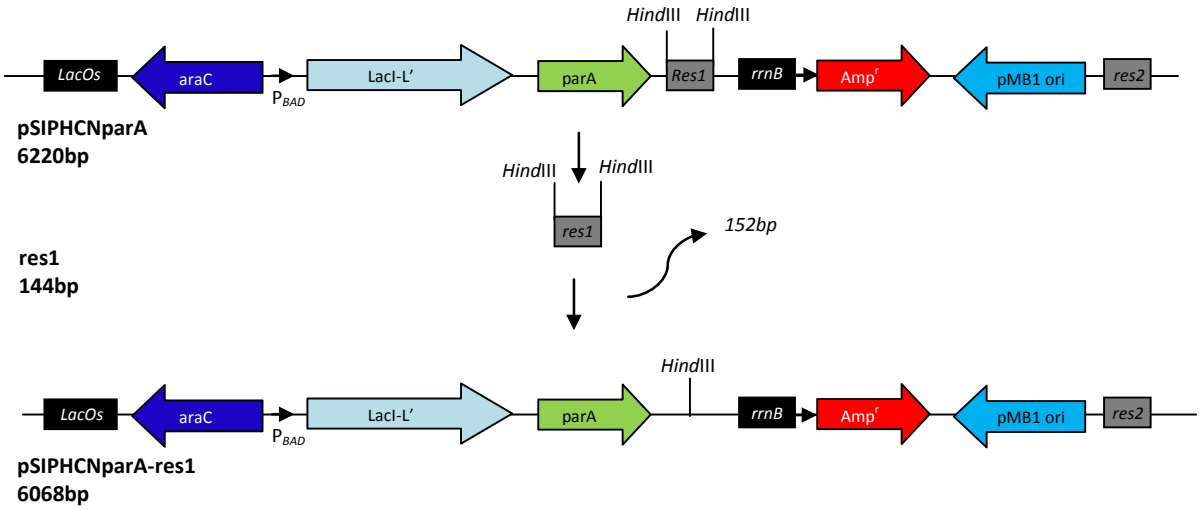


**Fig.1a.** ***Cloning strategy of plasmid pSIPHCNparA-res1;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

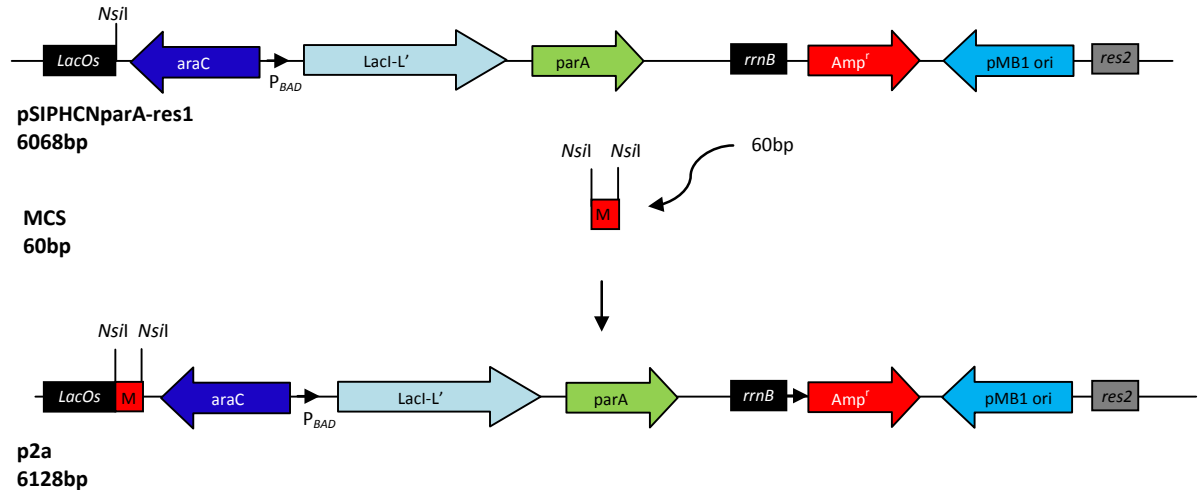


**Fig. 1b***: **Cloning strategy of plasmid p2a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
MCS1: 5'-ATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTACCTGCAGTTATAAGCTTATGCAT-3'
MCS2: 5'-TACGTAATTAATTGATCAGTCGAGTGCACGCCGGCGGGCCCATGGACGTCAATATTCGAATACGTA-3'

In order to allow the easy cloning of lethal gene (e.g. *I-Tev*II homing endonuclease that is needed for another study involving plasmid p3a) the expression cassette (LacI-L'/parA) should be inverted for tight repression and prevention of premature expression of cloned gene. *i.e.* before recombination. For this purpose plasmid p2a was digested with *Hind*III (Fermentas) which cuts the plasmid at two different locations and the plasmid was re-ligated using T4 DNA ligase (New England BioLabs, Germany) to get plasmid p3a (6128bp) (Fig. 1c).

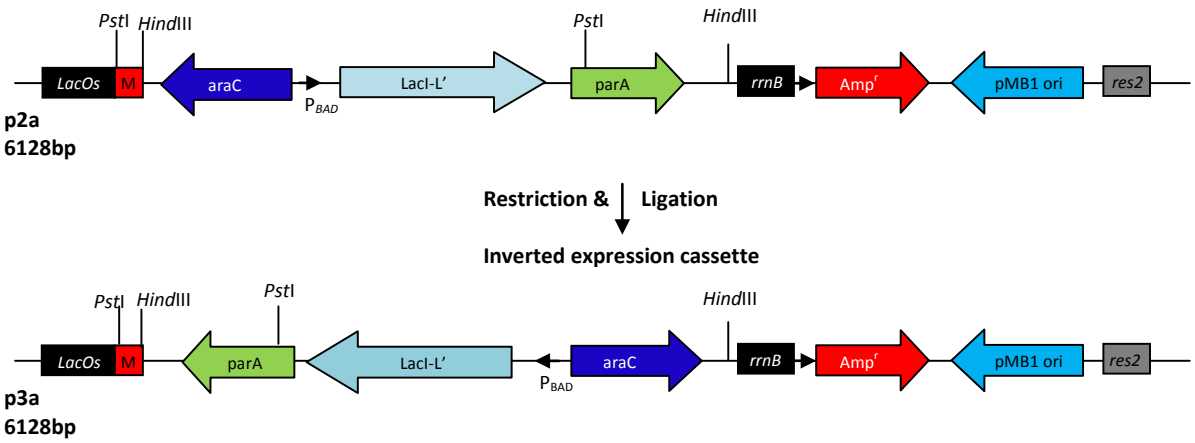


**Fig. 1c*: Cloning strategy of plasmid p3a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

### 3.2.2.2 Construction of plasmid p4a

To get the final working plasmid p4a (which has the ability to recombine) the resolution site1 was introduced into the plasmid p3a. For this purpose the 140bp res1 site was amplified through PCR using plasmid pSIPHCNparA [20] as template and primers 5'res1K (5'– CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3') and 3res1P (5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3) (synthesized by Microsynth AG, Switzerland) with *Kpn*I and *Psi*I restriction sites (underlined sequence in primer). The PCR reaction with final volume of 25µl was carried out using 0.25µl of (50 pmol/µl) primers each, 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) DreamTaq polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U/ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95°C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95°C for

30sec, 60°C for 30sec and 72°C for 1min and final elongation of 72°C for 10min. The PCR product was analyzed on 2% agarose gel to confirm the amplification. The PCR product was digested with *Kpn*I and *Psi*I (Fermentas) and subsequently cloned into the corresponding sites in plasmid p3a to get the vector p4a (6263bp) (Fig.2). The plasmid p4a is under control of pBAD promoter and has two resolution sites (res).

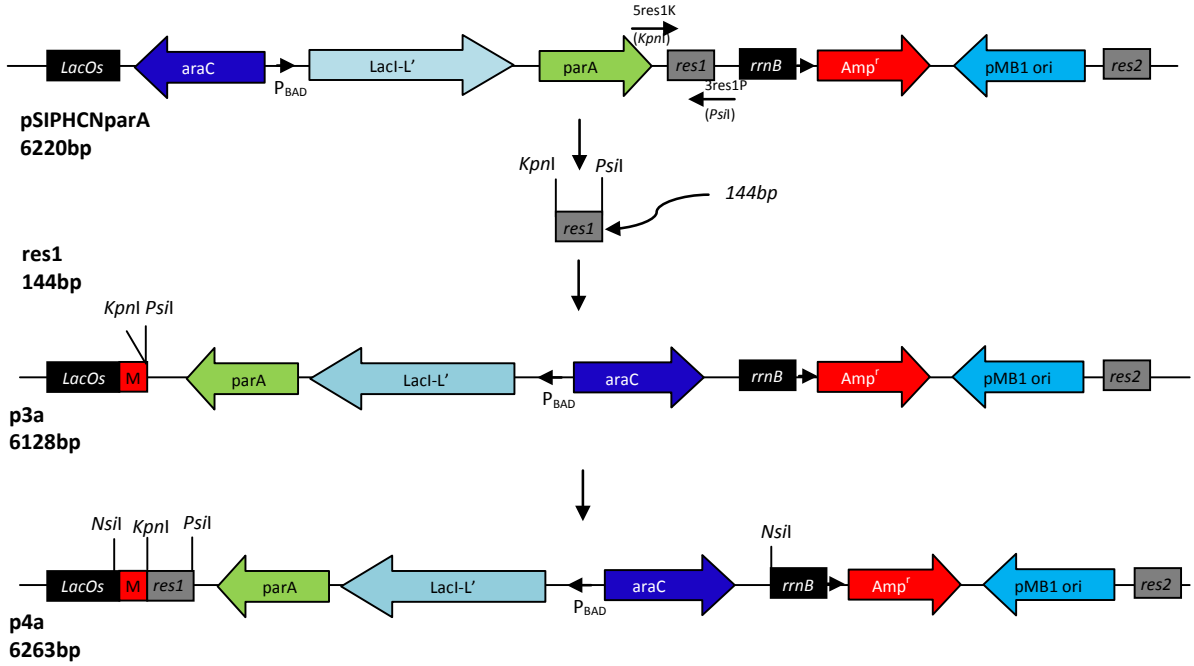


**Fig.2.** ***Cloning strategy of plasmid p4a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*5'res1K 5'– CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3'*
*3res1P 5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3*

### 3.2.2.3 Construction of plasmid p4aEYFP-C1

Before constructing plasmid p4aEYFP-C1 the removal of multiple cloning site from plasmid pEYFP-C1 (4731bp) (Fig.3) a kind gift by Dr. Monika Sramkova NIH America (Clontech, BD-Biosciences) was necessary. The plasmid pEYFP-C1 was digested and *Bgl*II and *BamH*I (both Fermentas) and 51bp MCS was removed and the plasmid was religated using T4DNA ligase (New England BioLabs, Germany) to get a new plasmid pEYFP-C1-MCS (4680bp) (Fig.3a).The 1596bp PCR fragment containing human cytomegalovirus (CMV) immediate early promoter (IE) and Enhanced yellow florescent protein (EYFP) gene along with polyA signals was amplified using primers 5'RYFP-*Sac*I 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3' and 3'RYFP-*Kpn*I 5'- ATA CCA GGT ACC TTA AGA

TAC ATT GAT GAG -3' (restriction sites underlined) and plasmid pEYFP-C1-MCS as template. The PCR condition was as follows. 25µl of total reaction containing 0.25µl of (50 pmol/µl) primers each (Microsynth AG), 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) Dream*Taq* polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of Dream*Taq* polymerase (Fermentas) at final conc. of (0.05U/ul).

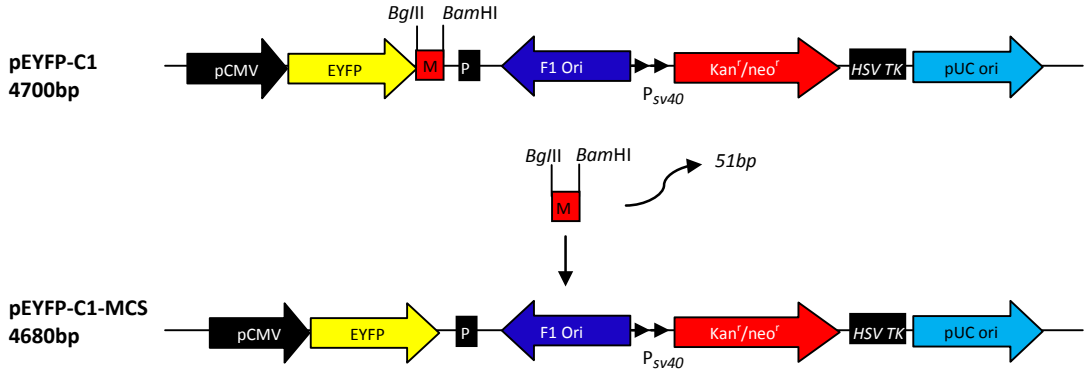


**Fig.3a.** ***Cloning Strategy of plasmid pEYFP-C1-MCS; for details see fig 3b.***

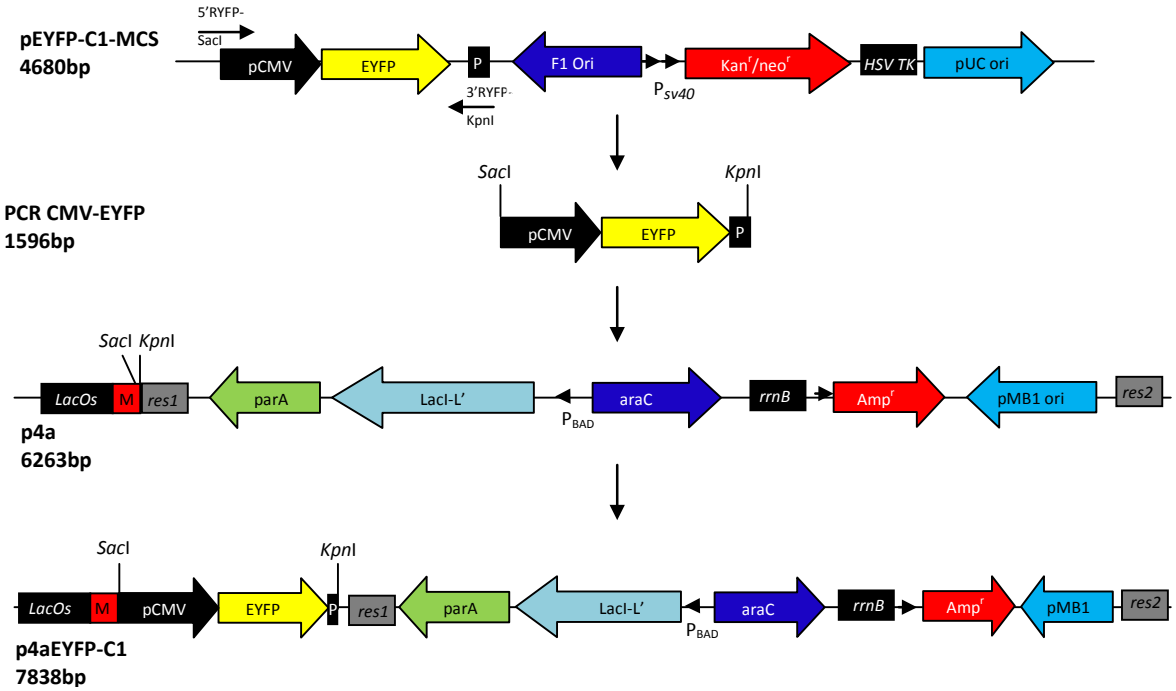


**Fig.3b.** ***Cloning strategy of plasmid p4aEYFP-C1;*** *pCMV, cytomegalovirus immediate early promoter; EYFP, enhanced yellow fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; Kan$^r$/neo$^r$; Kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95°C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95°C for 30sec, 58°C for 30sec and 72°C for 1min and final elongation of 72°C for 10min. The PCR product was analyzed on 2% agarose gel and subsequently digested with *Sac*I and *Kpn*I (Fermentas) and cloned into the corresponding sites in plasmid p4a resulting in vector p4aEYFP-C1 (7838bp) (Fig.3b).

### 3.2.2.4 Construction of plasmid p3aEYFP-C1

For cloning plasmid p3aEYFP-C1 (7703bp) the plasmid p3a was used as a vector. The plasmid p3a was digested with *Sac*I and *Kpn*I and the 1596bp PCR fragment amplified as described above in construction of plasmid p4a was ligated together into their corresponding sites after required manipulations to get plasmid p3aEYFP-C1 (Fig.3c).

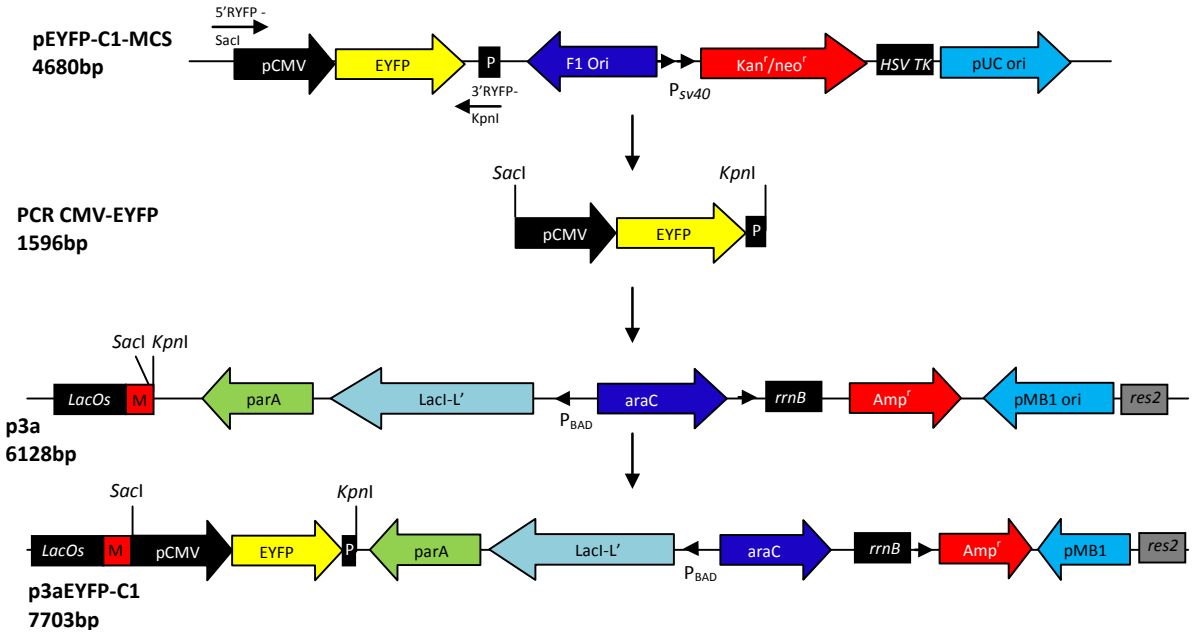


**Fig.3c.** ***Cloning strategy of plasmid p3aEYFP-C;*** *pCMV, cytomegalovirus immediate early promoter; EYFP, enhanced yellow fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; $Kan^r/neo^r$; Kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 3.2.2.5 Construction of plasmid p4amCherry-C1

The red fluorescent protein along with the eukaryotic promoter (CMV) and polyA sequence was cloned into the plasmid p4a through following procedure. The plasmid pmCherry-C1 (4722bp)(Fig.4a) a kind gift by Dr. Monika Sramkova NIH America (Clonetech, BD-Biosciences) was digested with *Bgl*II and *BamH*I (Fermentas) and re-ligated after removal of the multiple cloning site to get plasmid pmCherry-C1-MCS (4671bp) (Fig.4a)

The plasmid pmCherry-C1-MCS serves as a template for PCR amplification of 1587bp fragment containing Human cytomegalovirus (CMV) immediate early promoter (IE) and red fluorescent protein complex (CMV-mCherry) and polyA signal using primers, 5'RYFP-*Sac*I (5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3') and 3'RYFP-*Kpn*I (5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3') restriction sites underlined. The PCR reaction was same as described before for construction of plasmid p4aEYFP-C1. The PCR product and the plasmid p4a were subjected to restriction digest with *Sac*I and *Kpn*I (Fermentas) and were gel purified using Purelink$^{TM}$ Quick Gel extraction kit (Invitrogen). The 6263bp linearised vector fragment (p4a) was dephosphorelated with FastAP$^{TM}$ Thermosensitive Alkaline Phosphatase (Fermentas) to avoid re-ligation of the vector fragment. The PCR product was ligated with the plasmid p4a using T4 DNA ligase (New England Biolabs, Germany) to get the plasmid p4amCherry-C1 (7829bp) (Fig.4b).

### 3.2.2.6 Construction of plasmid p3amCherry-C1

The plasmid p3amCherry-C1 (7694bp) is similar to plasmid p4amCherry-C1 but lacks recombination activity. All the construction steps (except instead of plasmid p4a plasmid p3a) described for the construction of plasmid p4amCherry-C1 was used for cloning of plasmid p3amCherry-C1 (Fig.4c).

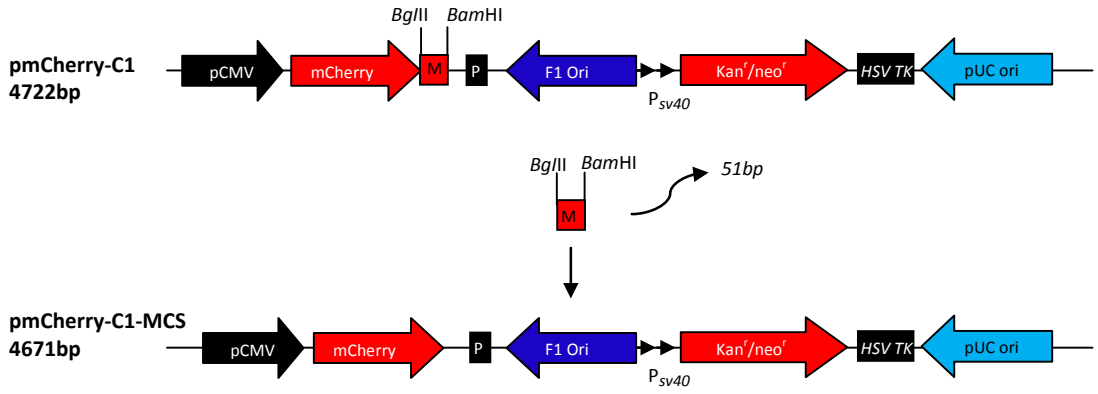


**Fig.4a.** ***Cloning strategy of plasmid pmCherry-C1-MCS; for details see Fig.4b***

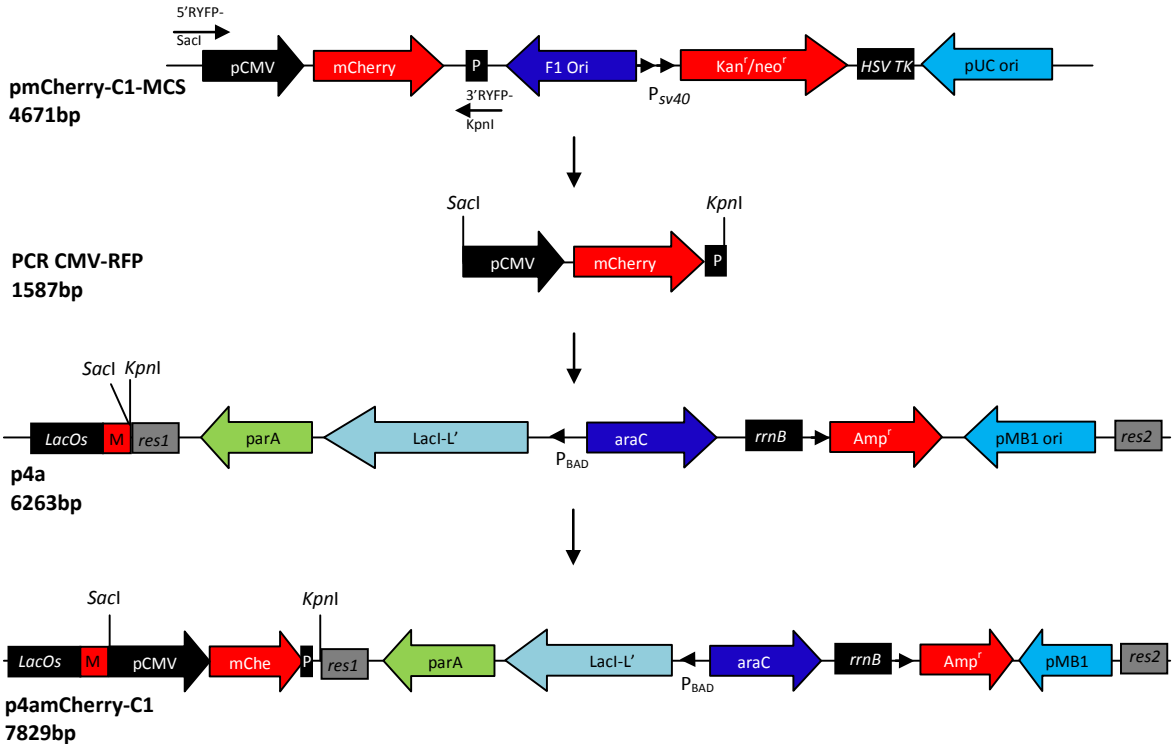


**Fig.4b.** ***Cloning strategy of plasmid p4amCherry-C1;*** *pCMV, cytomegalovirus immediate early promoter; mCherry, red fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; $Kan^r/neo^r$; kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

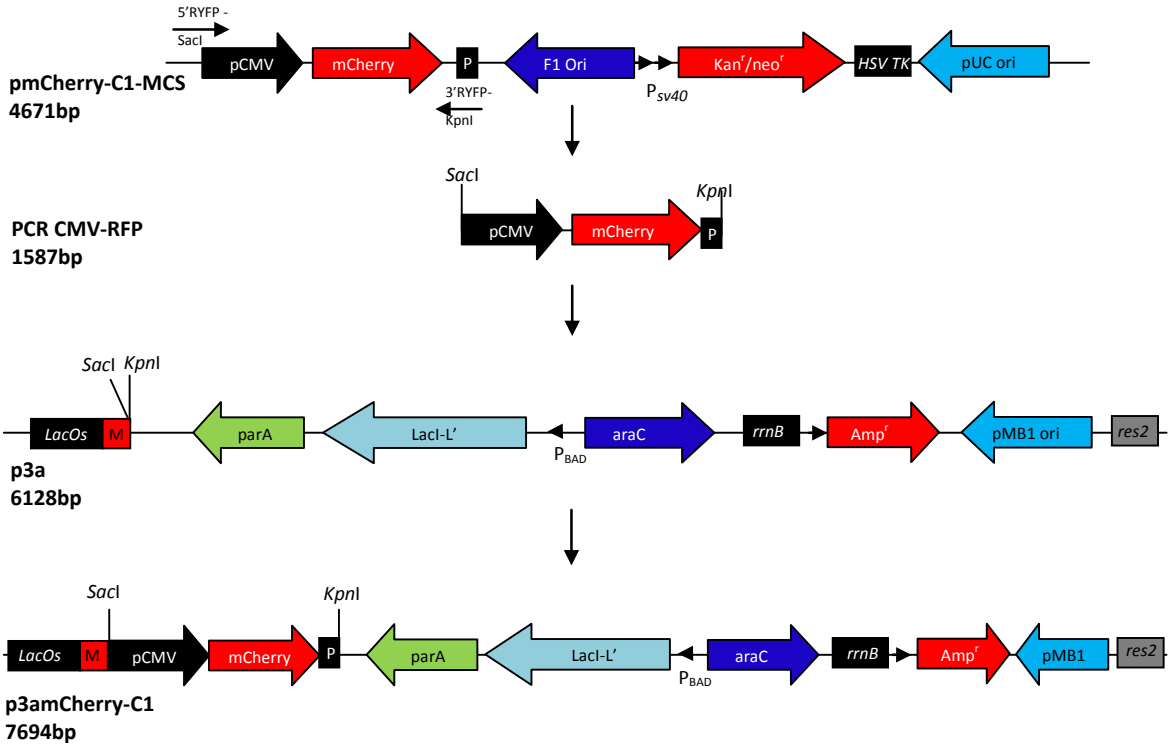


**Fig.4c.** ***Cloning strategy of plasmid p3amCherry-C1;*** *pCMV, cytomegalovirus immediate early promoter; mCherry, red fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; $Kan^r/neo^r$; kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; $Amp^r$, ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 3.2.2.7 Construction of plasmid p4aVenusA206K-C1

To construct plasmid p4aVenusA206K-C1 the removal of multiple cloning site from the plasmid pVenusA206K_C1 (4731bp) (Fig.5a) a kind gift by Dr. Monika Sramkova NIH America, was necessary. The plasmid pVenusA206K_C1 was digested with *Bgl*II and *BamH*I (Fermentas) and re-ligated resulting in plasmid pVenusA206K-C1-MCS (4680bp) (Fig.5a).

The 1596bp PCR fragment with *Sac*I and *Kpn*I restriction sites was amplified using pVenusA206K-C1-MCS as template using primers 5'RYFP-*Sac*I (5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3') and 3'RYFP-*Kpn*I (5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3') and was cloned in corresponding sites of plasmid p4a to get a

mcDNA carrying mammalian expression vector encoding an improved yellow-green fluorescent protein "Venus" p4aVenusA206K-C1 (7838bp) (Fig.5b).

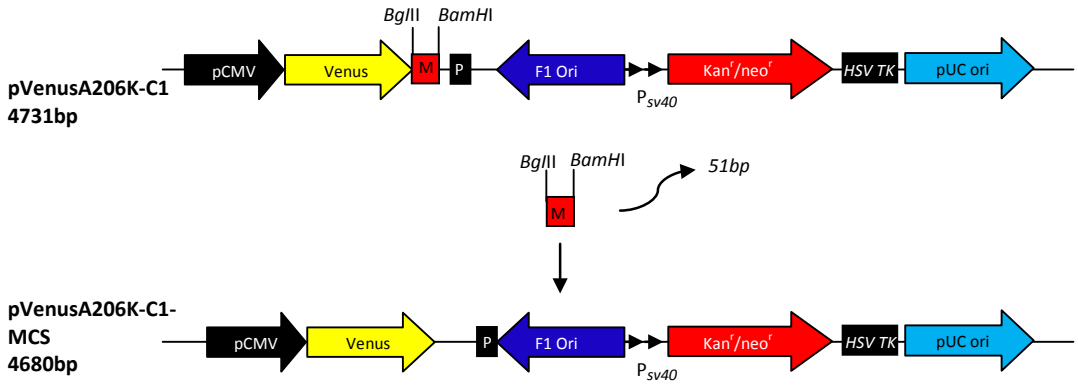


**Fig.5a.** ***Cloning Strategy of plasmid pVenusA206K-C1-MCS; for details see Fig.5b.***

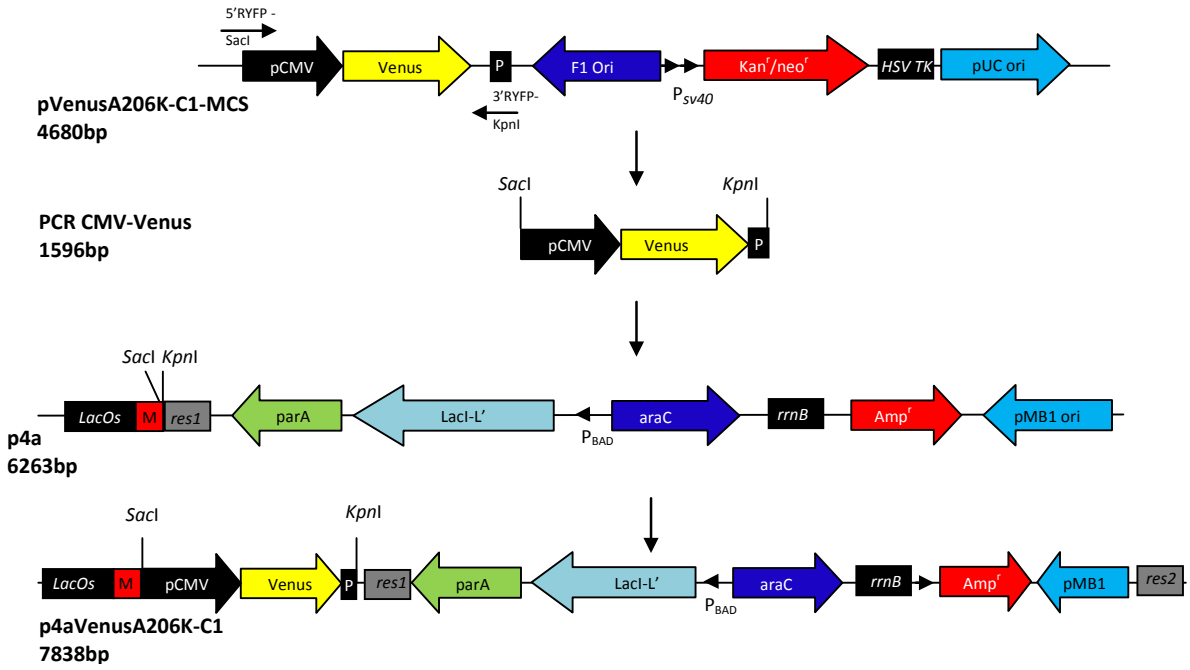


**Fig.5b.** ***Cloning strategy of plasmid p4aVenusA206K-C1;*** *pCMV, cytomegalovirus immediate early promoter; Venus, improved yellow-green fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; Kan$^r$/neo$^r$; kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*
*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 3.2.2.8 Construction of plasmid p3aVenusA206K-C1

For construction of plasmid p3aVenusA206K-C1 the plasmid pVenusA206K-C1-MCS was subjected to PCR amplification for the 1596bp CMV-Venus gene along with SV40 polyA tail flanked by *Sac*I and *Kpn*I using the primer set 5'RYFP-*Sac*I and 3'RYFP-*Kpn*I (for primer sequence and PCR procedure see construction of plasmid p4aVenusA206K-C1). Both the PCR product and plasmid p3a was digested with SacI and KpnI restriction enzymes (Fermentas) and were ligated at the correct vector to insert ratio after proper manipulation to obtain the plasmid p3aVenusA206K-C1 (7703bp) (Fig.5c).

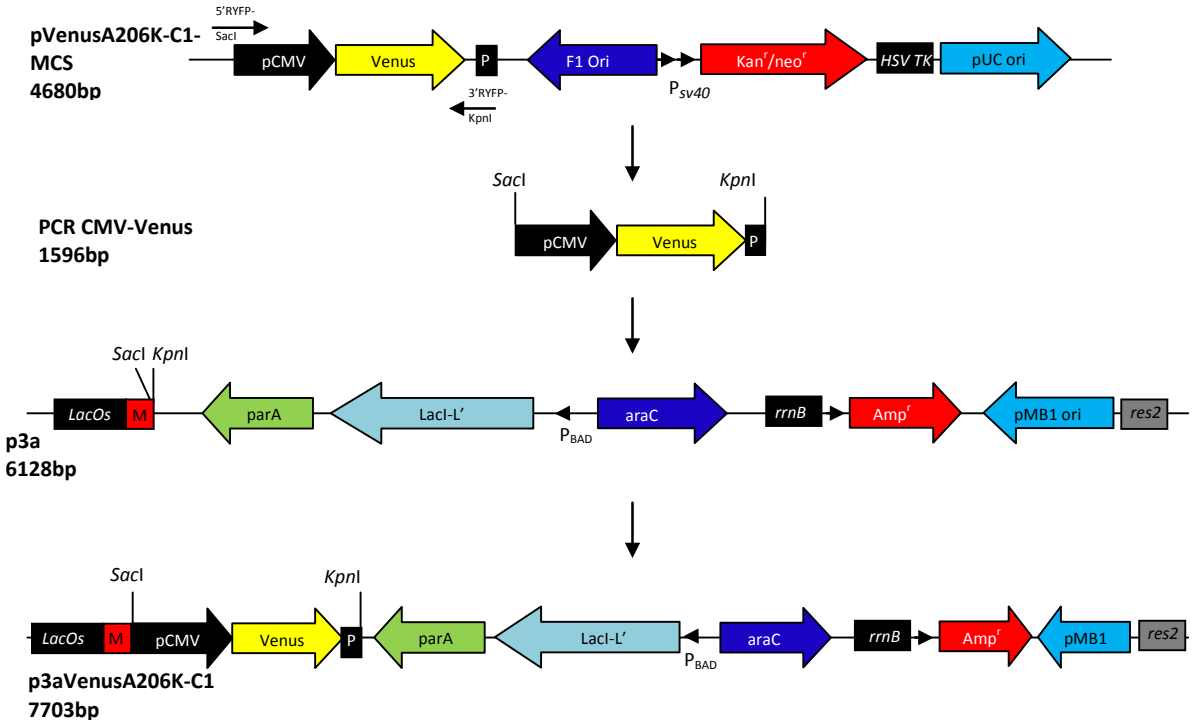


**Fig.5c.** ***Cloning strategy of plasmid p3aVenusA206K-C1;*** *pCMV, cytomegalovirus immediate early promoter; Venus, improved yellow-green fluorescent protein; M, multiple cloning site; P; polyadenylation signals derived from simian virus 40; F1 ori, phage derived origin of replication;, $P_{SV40}$: promoter derived from simian virus 40; Kan$^r$/neo$^r$; kanamycin and neomycin resistance cassette; HSV TK, Herpes simplex virus thymidine kinase polyadenylation signals; pUC ori; origin of replication derived from pUC19. lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

*Primers; 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'*

*3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3'*

### 3.2.3 Handling of plasmid DNA samples

Plasmid DNA samples were collected at different time points and plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). PureLink$^{TM}$ PCR purification Kit (Invitrogen) was used to purify the PCR products for further use. Whereas the PureLink$^{TM}$ Gel purification Kit (Invitrogen) was used to separate the DNA fragments or products from agarose gel all the above procedure were carried out according to the manufacturer's instruction. The transformation and electroporation of plasmid DNA was performed according to the standard molecular biological techniques by Sambrook *et.al.* [36]. The DNA modifying enzymes like T4 DNA ligase was purchased from New England Biolabs (Frankfurt Am Main, Germany), and the restriction enzymes from Fermentas (St. Leon-Rot, Germany). The PCR for amplification of DNA fragments was carried out using *Pfu* DNA polymerase and high Fidelity DNA polymerase also provided by Fermentas (St. Leon-Rot, Germany) 1µl of each primer, 2mM dNTPs (Fermentas) 10X$MgSO_4$ buffer. The PCR condition was set to 95$^{\circ}$C for 3 min followed by 35 cycles of 95$^{\circ}$C for 30sec 55$^{\circ}$C-60$^{\circ}$C (depending on Primer TM) for 30sec and 72$^{\circ}$C for 1min/kb product. Real time PCR for the quantification of the plasmid DNA in BGs and supernatant was carried out with iQ-SYBR Green super mix (Bio-Rad U.S.A). Both the PCR reactions were carried out in BioRad iCycler iQ$^{TM}$ Real-Time PCR detection system. For details of qPCR see (section 3.2.8).

### 3.2.4 Cell culture and reagents

The CCL-20.2 (Human conjunctival cell line) was kindly provided by Prof Bernd Binder (Medical University of Vienna, Austria). The cells were seeded 24 hour before transfection in a 24 well plate ($5x10^4$ cells per well) (SARATED, Ag & Co. Germany). Cells were maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 2mM glutamine (Invitrogen, Carlsband, CA), 100U/ml Penicillin (Invitrogen), 100µg/ml Streptomycin (Invitrogen), 10%FCS (Sigma Chemical Co., St. Louis, MO) and 10mM HEPES (Lonza) in a 5% $CO_2$ humidified incubator at +37$^{\circ}$C [37]. Prior to transfection the culture medium was replaced with fresh serum free culture medium. Transfections of cells were performed using different sets of plasmid DNA constructed for this work (see plasmid construction 2.2). Transfecting reagent TurboFect$^{TM}$ (Thermo Scientific, Austria) and 1µg/well of plasmid DNA used to transfect the cells (manufacturer's instructions followed). Cells incubated with or

without plasmid DNA and without transfecting reagent served as negative control. Transfection efficiencies were analyzed 24 and 48 hours later with the help of light fluorescence microscopy (Zeiss Axiovert S100 inverted microscope, Carl Zeiss, Jena GmbH, Germany).

### 3.2.5 Primer designing strategy

#### 3.2.5.1 Primer for quantification of mcDNA

Two sets of PCR primers were designed which when used in different combinations would then be able to quantify miniplasmid, midiplasmid and mother plasmid DNA. These primers are designed in a way that they will generate different size PCR products based on the physical forms (recombined or un-recombined) of same plasmid DNA used. For e.g. the primer McXF: 5'-GTG GTT TGT CCA AAC TCA TCA A-3' forward primer and McSR: 5'-ACA TGA GCA GAT CCT CTA CGC-3' reverse primer for quantification of mcDNA will generate two different size of PCR products i.e. 5443bp (un-recombined form) and 221bp (fully formed mcDNA). Melt curve analysis at the end of qPCR amplification is necessary to check the product size as larger DNA fragments has higher melting temperature. Presence of single sharp peak in melt curve graph is indication of the presence of single PCR product (Fig.5b).

#### 3.2.5.2 Primers for quantification of mpDNA

The primers designed to quantify the amount of retained miniplasmid in the pellet of BG preparations have similar properties to primers used for mcDNA quantification. Primer MpAF: 5'-TTT GCA AGC AGC AGA TTA CG-3' and MpYR: 5'-CGC AGC AGC AAA AAT AAA AG-3' is designed in a way that it is able to differentiate between the recombined and un-recombined mopDNA. The size of the PCR product in case of un-recombined mopDNA template will be 2401bp where as a 238bp product is generated when the plasmid is recombined (Fig.5c). A final melt curve is applied to check the specificity of the amplified product. Another advantage of above two primer sets is that they can be used to quantify the amount of mopDNA (Fig5a). In this case the forward primer designed for mcDNA quantification, McXF: 5'-GTG GTT TGT CCA AAC TCA TCA A-3' and reverse primer for mpDNA quantification, MpYR: 5'-CGC AGC AGC AAA AAT AAA AG-3' is used to

amplify 205bp fragment (only amplifiable in case of un-recombined mopDNA). The recombination efficiency (RE) of can be determined by quantifying the amount of mopDNA and replicative mpDNA at the end of reaction and calculated using following formulae after converting the ng of DNA into plasmid copy number using the formulae [copy number = (ng.of plasmid DNA x 6.022 x $10^{23}$) / (size of plasmid in bp. x 1 x$10^{9}$x 660)].

$$RE = 100 - \frac{\text{mopDNA copy number}}{\text{mpDNA copy number}}$$

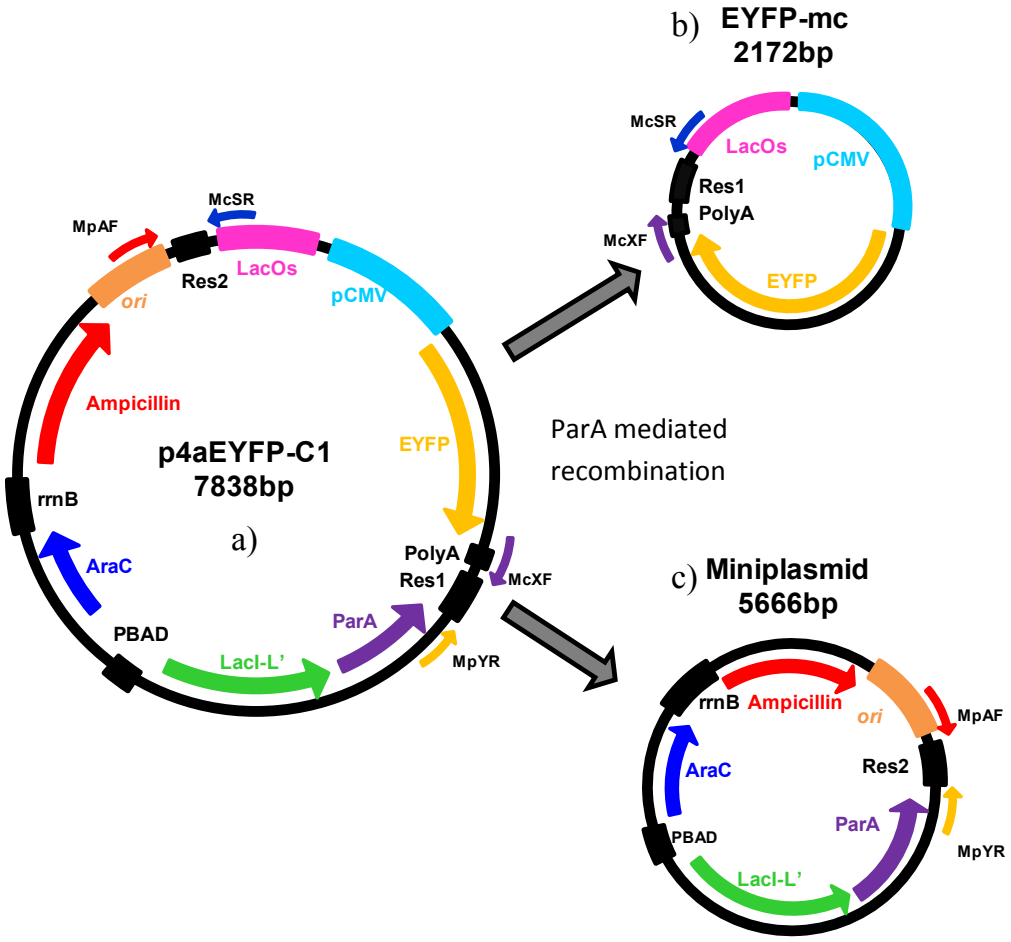


**Fig.5.** ***Detailed schema of the parA mediated site specific recombination;*** *production of mcDNA and primers binding sites for its quantification through qPCR technique. a) plasmid p4aEYFP-C1 (primer McXF and MpYR used to amplify mother plasmid); b) EYFP-mc (primer McXF and McSR used to amplify fully formed mcDNA); c) mpDNA (primer MpAF and MpYR used to quantify fully formed mpDNA);* ***araC,*** *repressor/inducer of $P_{BAD}$ promoter; EYFP, enhanced yellow fluorescent protein; LacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; LacOs, modified lac operator sequence with high affinity to bind lacI; ParA, ParA resolvase gene; $P_{BAD}$, arabinose inducible promoter; pCMV, cytomegalovirus early promoter; polyA, SV40 late poly adenylation signal; ori, origin of replication derived from pMB1 plasmid; Res1, resolution site 1; Res2, resolution site 2; rrnB, transcriptional terminator sequence of 5s ribosomal gene; MpAF, forward primer to amplify miniplasmid DNA region; MpYR; reverse primer to amplify miniplasmid DNA region; McXF, forward primer sequence to amplify minicircle DNA region; McSR, reverse primer sequence to amplify minicircle DNA region (for primer sequences see material and methods section.*

### 3.2.7 Production and purification of mcDNA and mpDNA for Real-time PCR Standards

In qPCR analysis the standard curve is the key to successful and accurate plasmid quantification which is dependent of plasmid preparations pure enough for dilution series pure plasmid DNA preparations has the ODA$260_{nm}/280_{nm}$ ratio of 1.8-1.95. To get purified mcDNA and mpDNA the mother plasmid p4aEYFP-C1 was freshly transformed into *E.coli* MC4100 cells and grown in 5ml LB supplemented with ampicillin (100µg/ml) and 2% glucose overnight ON at 36°C in a shaking incubator. A small part of this ON culture was shifted into nose flask containing 25ml LB supplemented with appropriate antibiotic (ampicillin 100µg/ml) (no glucose added) and grown in a 36°C water bath. The sample was continuously stirring at 300rpm until the $OD_{600}$ of 0.4-0.5 reached. The recombination was induced by addition of 0.25% L-(+)-arabinose. 1ml sample before and after the parA induction was collected and treated for plasmid DNA isolation through PeqLab Kit I (see handling of plasmid DNA) for densitometric analysis. After the 60 min of arabinose addition the cells were harvested by centrifugation at 10,000 rpm for 15min at 4°C. PureYeild™ plasmid Midiprep kit (Promega) is used to isolate the plasmid DNA. The samples were loaded on 1% agarose gel (Roth™) stained using gel red nucleic acid gel stain (GelRed™ Biotium # 41003) and analyzed under UV light in a ChemiDOC™ machine (BioRad laboratories). The mcDNA and mpDNA fragments were excised from gel and were isolated using PureLink™ Gel extraction Kit (Invitrogen). The visual quality of the preparation was checked on 1%agarose gel and purity through the UV absorption of A260/280 ratio using NanoDrop 2000c (peqlab).

### 3.2.7 Production of mcDNA loaded BGs

The plasmid p4aEYFP-C1,p4amCherry and p4aVenusA206k along with the plasmid pGLysivb [34] was transformed into *E.coli* MC4100. In plasmids with recombination ability the expression of fusion protein LacI-L' is kept under tight control of pBAD promoter while in plasmid pGLysivb the expression of lysis gene *E* is kept under temperature sensitive promoter/repressor system based on $\lambda p_{Rmut}/CI857$ [24]. The co-transformed *E.coli* MC4100 with above mentioned plasmids along with lysis plasmid pGLysivb was grown in 5ml LB supplemented with appropriate antibiotics ampicillin and gentamycin at different concentrations (100µg/ml and 20µg/ml respectively) along with 2% glucose in shaking incubator at 36°C ON. A part of this ON culture is shifted to 100ml nose flask containing ampicillin (100µg/ml) and gentamycin (20µg/ml) and no glucose. The sample was grown in

36°C water bath at continuous stirring of 300rpm until OD 0.15-0.2 was achieved. The expression parA resolvase and of LacI-L' fusion protein is induced by the addition of L-(+)-arabinose at final concentration of 0.25% resulting in recombination of mopDNA into mpDNA and mcDNA the later is immobilization inside the bacterial membrane via LacI-L' anchor protein. 30min after induction of pBAD promoter the lysis gene *E* is induced by shifting the flask from 36°C to 42°C water bath and stirring at 300rpm. The lysis was continued and monitored for another 2 hours through optical density observation $OD_{600}$, physical observation of bacterial cells done through light microscopy and determination of viable bacterial cells was carried out through platting culture on plain LB plates using automated system spiral platter (WSAP system; DON Whitley Scientific Limited, West York Shire, UK). The LB plates were grown at 36°C over night. The colonies were counted using colony counter machine 3.15 (Synoptic Ltd., Cambridge, UK) using the program Synbiosis ProtoCOL.

### 3.2.8 Sample preparation and quantification of mcDNA/mpDNA through qPCR

For quantification of mcDNA and mpDNA at different time points 2 ml of the culture medium is collected in 2ml eppendorf tubes and centrifuged at 13000rpm for 3 min. the 1ml of the supernatant was filtered using 0.2µm sterile cellulose acetate membrane filters (VWR, international) and treated for plasmid DNA isolation (see section 3.2.3). The qPCR was performed in iQ 96 well transparent PCR plates (Bio-Rad) containing 12.5µl of iQ-SYBR Green super mix (Bio-Rad) 1µM of each primer (Microsynth AG), 5µl of template DNA in a final volume of 25µl. Microseal 'B' (Bio-Rad) adhesive based sealing was used to seal and prevent the sample evaporation from the plate. Following cycling condition was applied to quantify the mcDNA or mpDNA, 95°C for 3min, followed by 30 cycles of 94°C for 40sec and 60°C for 60sec., the fluorescence signal was recorded during each extension step. BioRad iCycler iQ Multiple-color Real Time PCR detection system was used for performing the whole procedure. 10 fold serial dilutions $10^{-1}$ -$10^{-6}$ of purified mcDNA and mpDNA (section 3.2.7) with known concentration were used to perform standard curve analysis for final quantification. In order to check the specificity of PCR product amplified the sample was subjected to a final step of melting gradient form 60°C to 95°C with interval of 10sec. and checked on 2% agarose gel. For primers sequences please check (section 3.2.5). The whole experimental procedure was carried out according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [38].

## 3.3 Results

### 3.3.1 Transfection efficiency of CCl-20.2 cells

To check the expression of eukaryotically regulated cloned genes in newly constructed plasmids (p4aEYFP-C1 (Fig.3b), p3aEYFP-C1 (Fig.3c), p4amCherry-C1 (Fig. 4b), p3amCherry-C1 (Fig.4c), p4aVenusA206K-C1 (Fig.5b), p3aVenusA206K-C1(Fig.5c)) the plasmids were tested in CCL-20.2 cell line. The cells were transfected using TurboFect$^{TM}$ and in combination with above mentioned plasmids and the plasmids from which the fluorescent genes were taken and cloned in them i.e. pEYFP-C1 (Fig.3a), pmCherry-C1 (Fig.4a) and pVenusA206K_C1 (Fig.5a). The light fluorescent microscopy of samples after 48hours of transfection revealed no fluorescence in cells incubated with the plasmid DNA alone (Fig.6. a1, a2 and a3). Medium to high fluorescence signals were detected in plasmid p3aEYFP-C1 and p4aEYFP-C1 respectively (Fig.6. c1 and d1) compared to the plasmid pEYFP-C1 (Fig.6. b1) from which Enhanced Yellow Fluorescent Protein has been cloned into former two plasmids. Apparently no change in fluorescence intensity was observed in cells transfected with plasmid preparations from pmCherry-C1, p3amCherry-C1 and p4amCherry-C1 (Fig.6. b2, c2 and d2 respectively). In case of Venus which is a mutated green fluorescent protein higher fluorescent signals were detected in cells transfected with plasmid p3aVenusA206K-C1 (Fig.6. c3) compared to the origin plasmid pVenusA206K_C1 (Fig.6. b3) where as the fluorescence intensity in cells incubated with p4aVenusA206K-C1 was medium as compared to the above two plasmids of the same group (Fig.6. d3). The above transfection experiment shows that the fluorescence signals of the reporter gene was enhanced by their subsequent cloning into plasmid p4a and p3a where as among them the best fluorescence was recorded in plasmid p3aVenusA206K-C1 and p4aEYFP-C1.

**Table.1. Relative Intensity of different plasmid constructs**

| | Enhanced yellow fluorescent protein | | | Red fluorescent protein | | | Yellow green fluorescent protein | | |
|---|---|---|---|---|---|---|---|---|---|
| Relative intensity | EYFP-C1 | p3aEYFP-C1 | p4aEYFP-C1 | mCherry-c1 | p3amCherry-c1 | p4amCherry-c1 | VenusA206K_C1 | p3aVenusA206K-C1 | p4aVenusA206K-C1 |
| | +(+) | ++(+) | ++++ | ++(+) | ++(+) | ++(+) | ++ | ++++ | +++(+) |
| Amount of plasmid DNA used | 1µg | | | 1µg | | | 1µg | | |

CCl-20.2 cells transfected with different plasmid constructs with TurboFect$^{TM}$

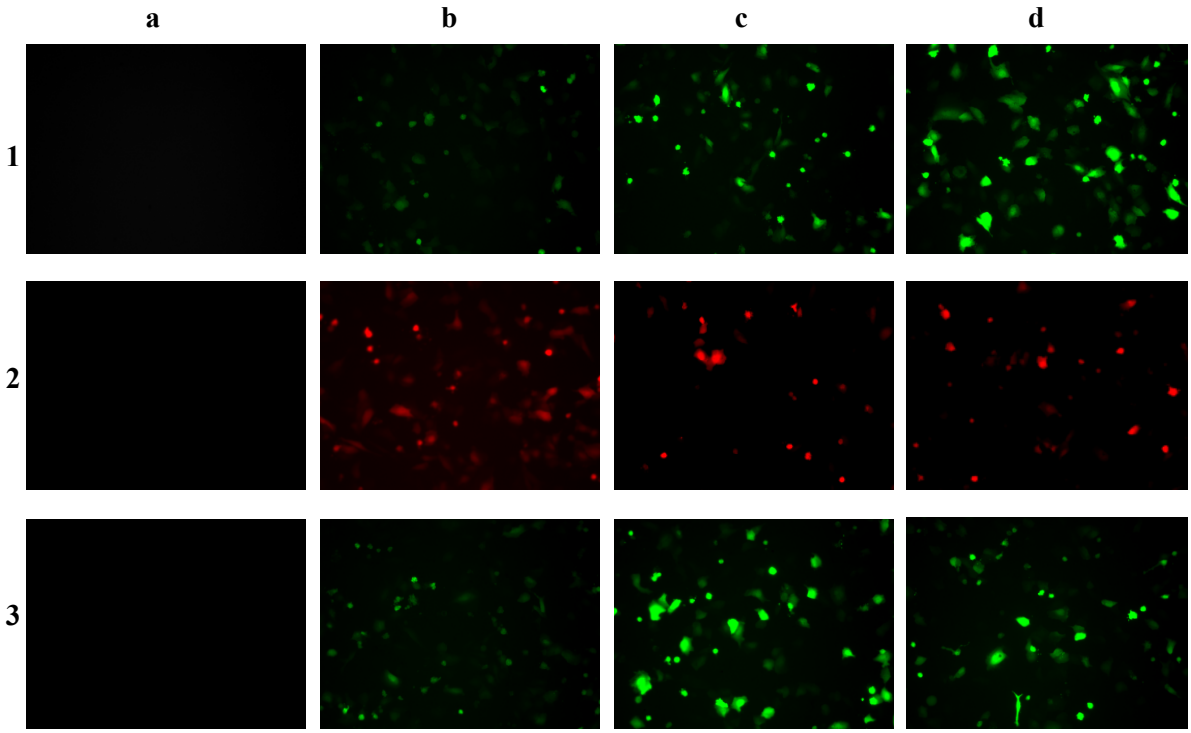


***Fig.6.Transfection of CCL-20.2 cells;*** *Transfection of CCL-20.2 cells resulted in better fluorescence of cloned; enhanced yellow fluorescent protein EYFP, Red fluorescent protein RFP and VenusA206K green fluorescent proteins 48 hours post transfection. 1a) Control, 4x10$^4$ cells plus naked DNA; 1b) pEYFP-C1 plus TurboFect$^{TM}$; 1c) p3aEYFP-C1plus TurboFect$^{TM}$; 1d) p4aEYFP-C1plus TurboFect$^{TM}$ ; 2a) Control, 4x10$^4$ cells plus naked DNA; 2b) pmCherry-C1 plus TurboFect$^{TM}$; 2c) p3amCherry-C1plus TurboFect$^{TM}$; 2d) p4amCherry-C1plus TurboFect$^{TM}$; 3a) Control, 4x10$^4$ cells plus naked DNA; 3b) pVenusA206K_C1 plus TurboFect$^{TM}$; 3c) p3aVenusA206K-C1plus TurboFect$^{TM}$; 3d) p4aVenusA206K-C1plus TurboFect$^{TM}$*

### 3.3.2 Production and purification of mcDNA by site-specific ParA recombination

ParA recombination was induced by the addition of L-(+)-arabinose in *E.coli* MC4100 harboring plasmid p4aEYFP-C1, p4amCherry-C1 and p4aVenusA206K. Complete recombination is seen in plasmid p4aEYFP-C1 after 120 min of induction (Fig.7. lane 3) while no recombination is observed before the arabinose induction. (Fig.7. lane 2). Supplementation of 2% glucose inhibits premature recombination in ON culture (Fig.7. lane 1). Harvested cells were subjected to DNA isolation and were purified for use in Real-time PCR standards (see section 3.2.3). Clear bands of mcDNA and mpDNA can be seen at desired positions after the gel purification (Fig.8).

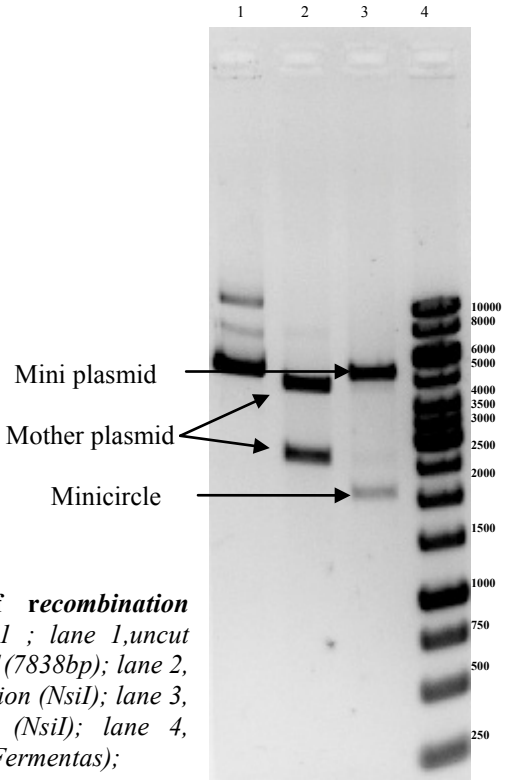


**Fig.7. Restriction analysis of r*ecombination product;*** *plasmid p4aEYFP-C1 ; lane 1,uncut over night culture p4aEYFP-C1(7838bp); lane 2, time point A before recombination (NsiI); lane 3, 60min after recombination (NsiI); lane 4, GeneRuler™ 1kb DNA ladder(Fermentas);*

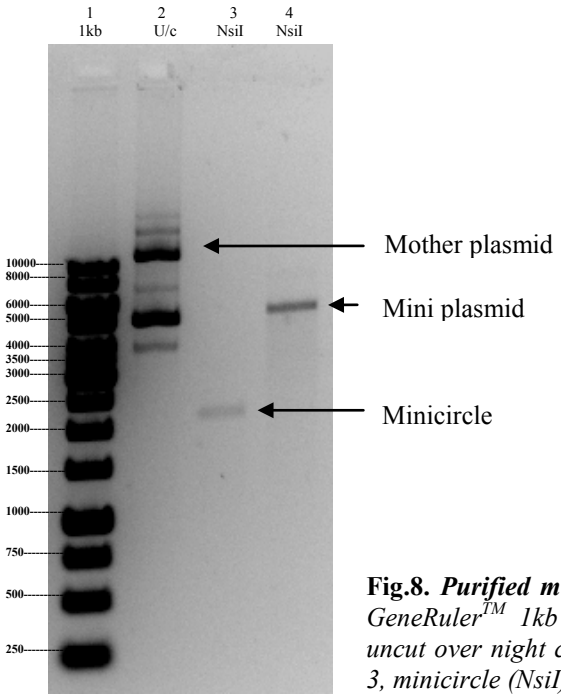


**Fig.8. *Purified minicircle and miniplasmid***; *lane 1, GeneRuler™ 1kb DNA ladder(Fermentas); lane 2 uncut over night culture p4aEYFP-C1(7838bp); lane 3, minicircle (NsiI); lane 4,Miniplasmid (NsiI)*

### 3.3.3 Production of immobilized mcDNA loaded BGs

The plasmid p4aEYFP-C1 (Fig.3b) has all necessary genetic elements for its recombination into mcDNA and mpDNA and its immobilization inside the bacterial membrane. The LacI-L' fusion protein is expressed within the bacterial strain *E.coli* MC4100 carrying plasmid p4aEYFP-C1. The resolvase gene is also under the expressional control of pBAD promoter which upon induction recombines the mopDNA into mcDNA and mpDNA, of which the former carries the lacOs site responsible for its immobilization inside the BGs by interacting with LacI-L' anchor protein. In-order to produce the bacterial ghosts carrying these immobilized mcDNA a second plasmid system pGLysivb [39] carrying lysis gene *E* under the control of temperature sensitive promoter/repressor system is co-transformed inside the bacterial cells. No inhibition in growth of bacterial cells recorded upon induction of pBAD promoter (Fig.9a.). The microscopic picture of cells did not show any morphological changes after the addition of L-(+)-arabinose (Fig.9b.i) and about 50 min after the induction of pBAD promoter the mcDNA is visible on gel (Fig.9c. lane 4) upon induction of lysis gene *E* the cytoplasmic content is expelled from the bacterial cells. A light microscopic picture of BG can be seen in (Fig.9b. ii). Loss in lysis plasmid and mopDNA where as decrease in amount of mpDNA can be seen in (Fig.9c. lane 4, 5, 6 and 7).

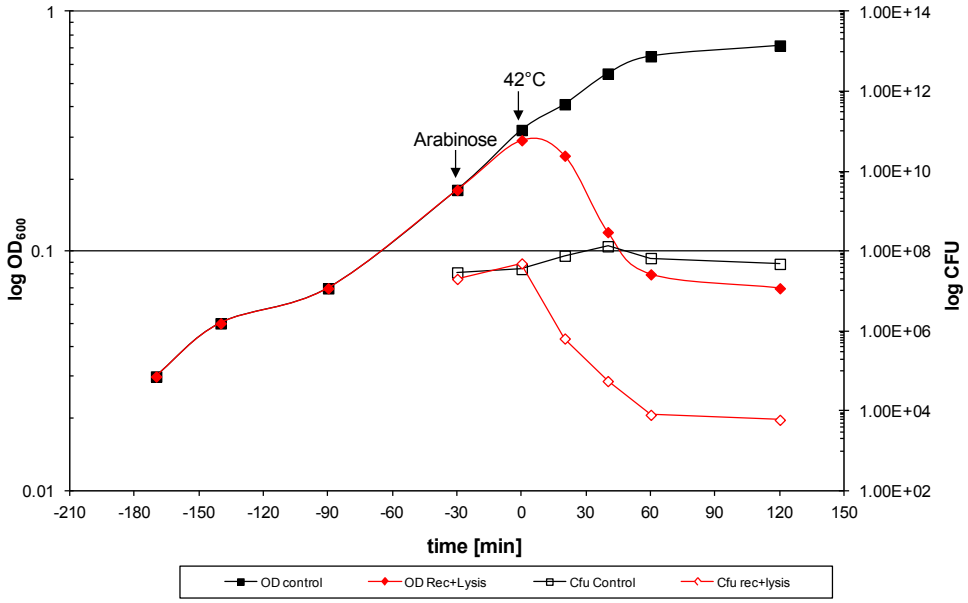


**Fig.9a.** ***Expression of ParA gene and lysis curve of plasmid p4aEYFP-C1;*** *OD and CFU count of recombination lysis test. E.coli* MC4100 *carrying plasmid p4aEYFP-C1+pGLysivb. Black line is the control. Drop in OD and CFU is recorded in samples treated with L-(+)-arabinose (0.25%) at time point -30mi and induction of gene E (Red line) a lysis efficacy of 99.99%. -30'min is the time point of arabinose where as 0' min is the time pint of lysis induction.*

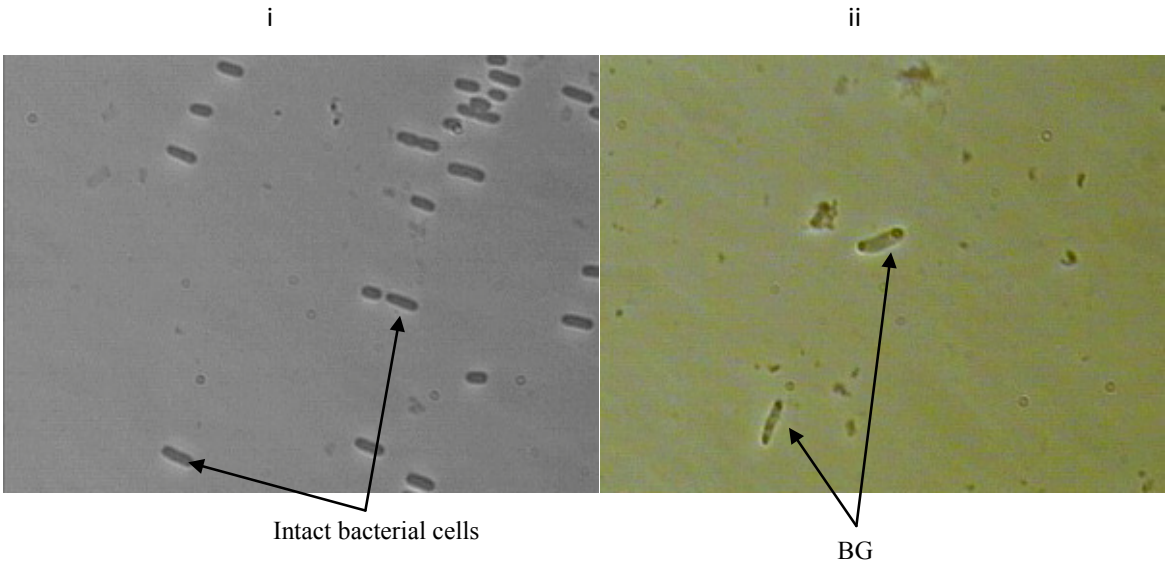


**Fig.9b.** ***Microscopic picture of MC4100Cell harboring plasmid p4aEYFP-C1 and pGLysivb; i)*** *intact cells growing normally after recombination and before lysis induction;* ***ii)*** *Bacterial Ghosts, 120 min after gene E induction.*

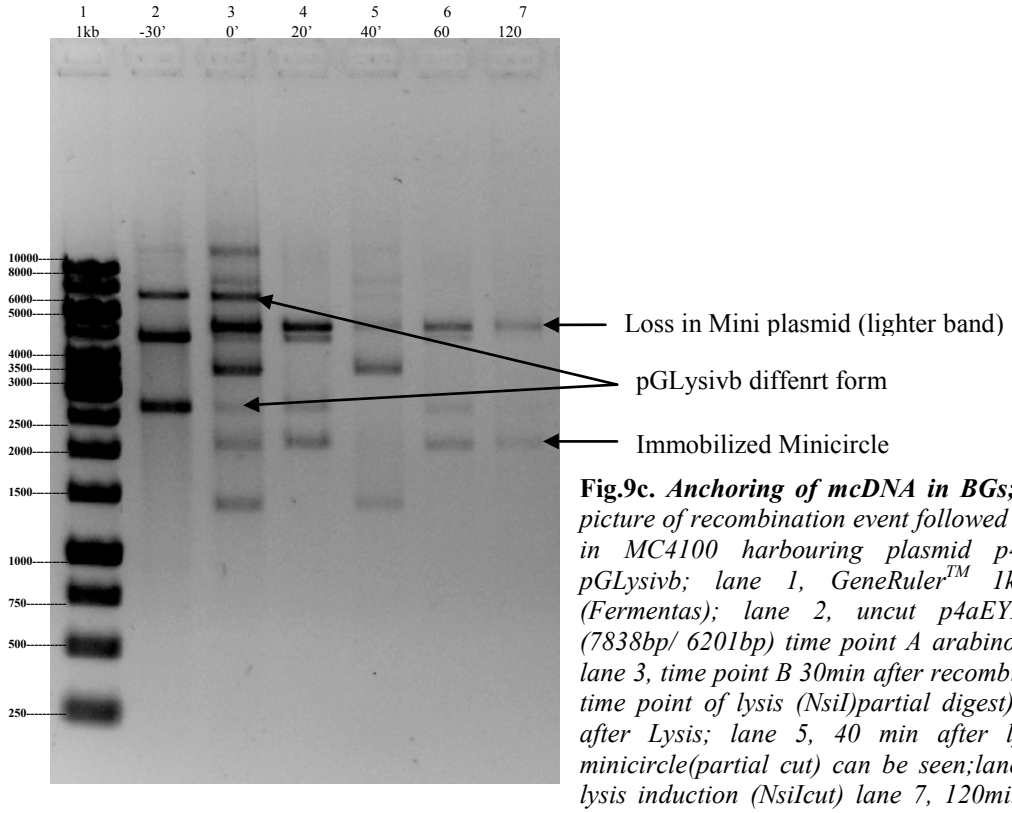


**Fig.9c.** ***Anchoring of mcDNA in BGs;*** *1% agarose gel picture of recombination event followed by lysis induction in MC4100 harbouring plasmid p4aEYFP-C1 and pGLysivb; lane 1, GeneRuler™ 1kb DNA ladder (Fermentas); lane 2, uncut p4aEYFP-C1+pGLysivb (7838bp/ 6201bp) time point A arabinose addition time; lane 3, time point B 30min after recombination and 0'min time point of lysis (NsiI)partial digest) ; lane 4, 20min after Lysis; lane 5, 40 min after lysis immobilized minicircle(partial cut) can be seen;lane 6, 60 min after lysis induction (NsiIcut) lane 7, 120min after lysis mini circle is immbobilised inside the ghost*

### 3.3.4 Qualitative PCR detection of desired sequences of mcDNA / mpDNA

Qualitative PCR was carried out to determine the specificity of used primers. The qualitative PCR was performed as described in material and methods (section 3.2.3). A 221bp fragment of fully formed mcDNA was amplified with the primer McXF and McSR (for primer sequence see section 3.2.5) when a recombination product from plasmid p4aEYFP-C1 was used as a template (Fig.10a lane 5), the same primer yielded product of varying size when un-recombined plasmid or mother plasmid p4aEYFP-C1 was used as template (5887bp and 221bp product suggesting partial recombination) (fig.10a lane 4). No DNA band at 221bp region seen on 2% agarose gel in case of p3aEYFP-C1 (fig.10a. lane 3) due to the absence of resolution site1 (res1).

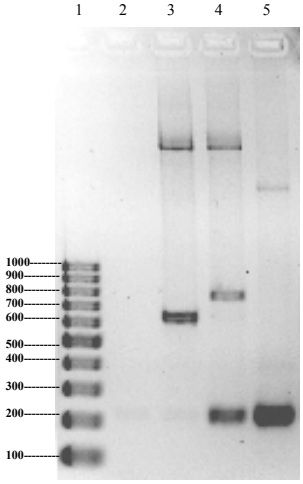


**Fig.10a.** ***Amplification of mcDNA;*** *2% agarose gel picture of PCR product of different regions of plasmid p4aEYFP-C1 and p3aEYFP ; lane 1, GeneRuler™ 100bp DNA ladder (Fermentas); lane 2, negative control for minicircle DNA primers; lane 3, plasmid p3aEYFP-C1; lane 4, plasmid p4aEYFP-C1 221bp if recombined and un-recombined 5887bp(sample partially recombined); lane 5, plasmid p4aEYFP-C1 recombined (221bp fragment only)*

The primers designed for amplification of mpDNA MpAF and MpYR (for sequence see section 3.2.5) gave a clear band of 238bp size when recombined plasmid p4aEYFP-C1 was used as a template (Fig.10b.lane 11 and 12). Whereas the above primer set amplified 2401bp region when un-recombined plasmid p4aEYFP-C1 was used as template (Fig.10b. lane 10), a faint band at 238bp region suggests slight recombination. A simple PCR with plasmid p3aEYFP-C1 yielded a 2275bp product when primer MpAF and MpYR was used (Fig.10b. lane 9). The above two primer sets are designed in a way that if the forward primer of one (McXF) and the reverse primer of second (MpYR) is used in a reaction; it will only amplify the mopDNA (Fig.5). This is because if the plasmid is recombined the two region will be distributed among the daughter plasmids i.e. mcDNA and mpDNA. This primer combination amplified 71bp region on plasmid p3aEYFP-C1 (Fig.10b. lane 3) and 205bp

region on plasmid p4aEYFP-C1. No amplifications seen when lysis plasmid pGLysivb and pGLNIc was used as template (Fig.10b. lane 7 and 13 respectively).

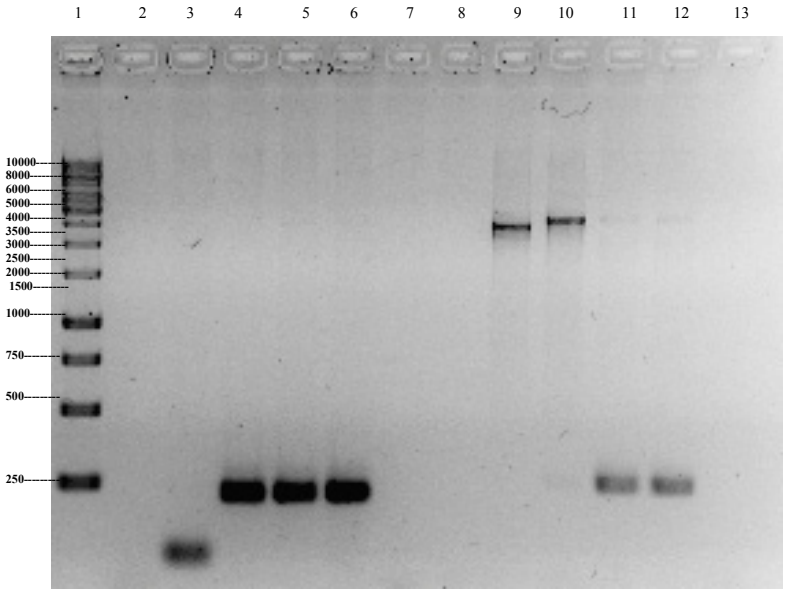


**Fig.10b. Analysis of *PCR product of motherplasmid and miniplasmid DNA;*** *2% agarose gel picture of PCR product of different regions of plasmid p4aEYFP-C1 and p3aEYFP ; lane 1, GeneRuler$^{TM}$ 1kb DNA ladder (Fermentas); lane 2, negative control for mop DNA primers; lane 3, plasmid p3aEYFP-C1 71bp; lane 4,5and 6 plasmid p4aEYFP-C1 205bp for mop; lane 7, pGLysivb; lane 8, negative controll for mpDNA primer; lane 9, p3aEYFP-C1 2275bp region; lane 10, p4aEYFP-C1 unrecombined (2401bp region); lane 11 and 12, p4aEYFP-C1 (recombined) 238bp fragment ; lane 13, pGLNIc.*

### 3.3.5 Quantification of mcDNA and mpDNA

Samples for mcDNA/mpDNA quantification in pellet and supernatant of the lysis culture were prepared as described in (section 3.2.8). The samples from the recombination and lysis experiments were collected at different time points and subjected to qPCR for mcDNA/mpDNA load (Fig.11). Induction of parA resolves gene at time point -30'min results in recombination and the amount of minicircle can be seen increasing in the pellet and stays constant even after lysis of cell suggesting the anchoring of mcDNA via LacI-L' anchor protein to the bacterial inner membrane (Fig.11a). The amount of mcDNA and mpDNA in the supernatant increases upon lysis induction time point 0'min (Fig.11b and d) this release is due to the lysis tunnel formed by fusion of inner and outer membrane through which most of the cytoplasmic content is released [26].

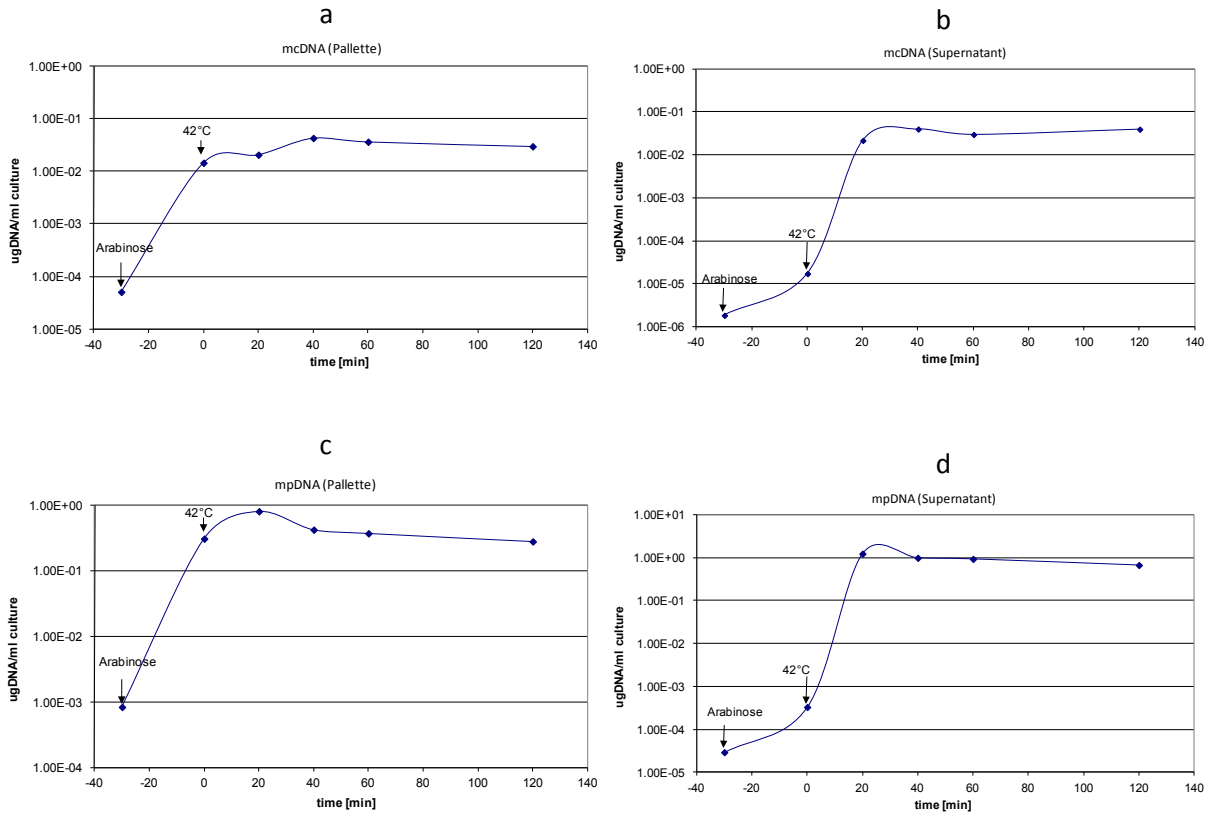


**Fig.11.** ***Monitoring of recombination and lysis process through real time PCR;*** *Pattern of mcDNA / mpDNA concentration at different time points;* ***a)****mcDNA increase after addition of arabinose in pellet;* ***b)*** *amount of mcDNA in the supernatant of culture medium which increases after lysis induction time point 0'min;* ***c)*** *Amount of mpDNA in the pellet of the culture medium;* ***d)*** *concentration of mpDNA in the*

After this E lysis majority of the mpDNA along with mopDNA is expelled whereas along with this some amount of mcDNA is also expelled but still most of the mcDNA is retained inside the BG through L'anchor this change in DNA content is recorded through qPCR and the percentage loss of mcDNA and mpDNA during lysis process is shown in graph (Fig.12a). The percentage of mcDNA recovered from the pellet of the lysed culture was 61% where as the rest is mpDNA. Similarly the percentage of mpDNA was found to be higher in the supernatant (Fig.12b). The amount of mcDNA and mpDNA isolated from pellet of 1ml culture quantified through qPCR after conversion into number of mcDNA / BGs and mpDNA / BGs from two different experiments is presented in (Table.2).

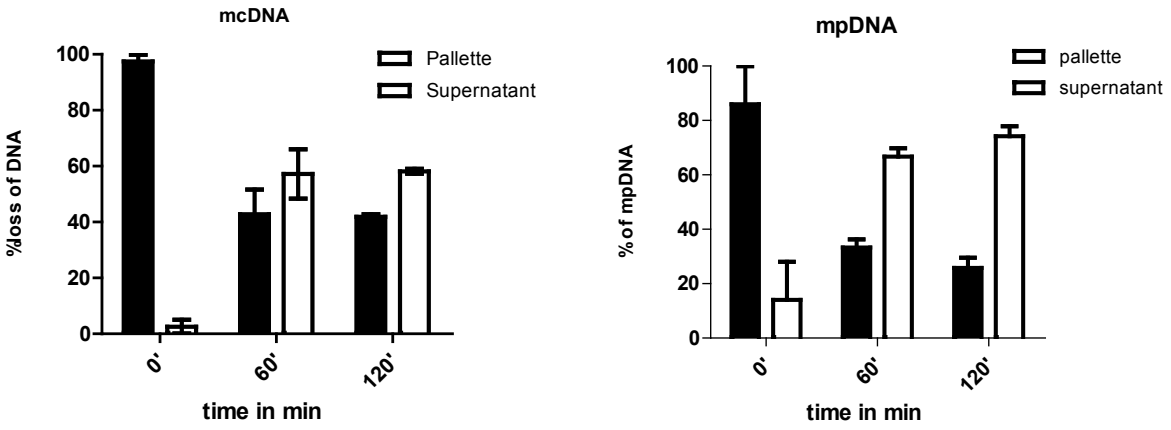


**Fig.12a.** ***Graphical presentation of amount of mcDNA and mpDNA in BGs;*** *percentage of total mcDNA (left) and mpDNA (right) at different time points of lysis quantified through qPCR; Loss in mpDNA can be seen in pellet after 120 min of lysis (right) while a considerable amount of mcDNA is still retained in the pellet after 120min of lysis (left); mean SD of two different recombination lysis experiments.*

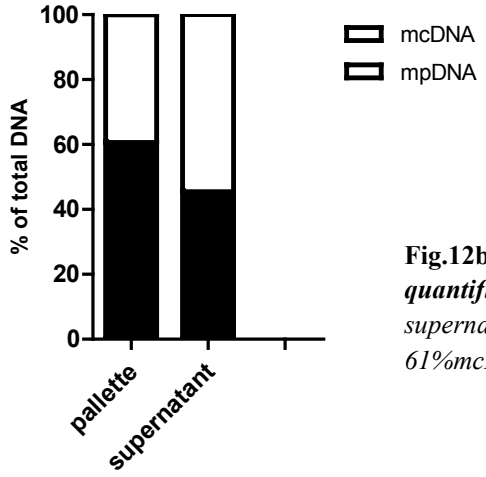


**Fig.12b.** ***Graphical presentation of total amount of DNA quantified;*** *percentage of mcDNA and mpDNA in pellet and supernatant of the lysed culture calculated through qPCR; 61%mcDNA in pellet (dark part) 39%is mpDNA (empty part)*

**Table.2. Amount of mcDNA and mpDNA retained inside the BGs**

| mcDNA ng. / total no of cells | mcDNA copy /BG | mpDNA ng. / total number of cells | mpDNA copy / BG |
|---|---|---|---|
| 13.5ng / $1.19x10^7$ | 479.7 | 27.3ng / $1.19x10^7$ | 371 |
| 39.0ng / $4.32x10^7$ | 378.5 | 133ng / $4.32x10^7$ | 495.78 |

Results from two different recombination lysis experiments showing amount of minicircle DNA (mcDNA) and miniplasmid DNA (mpDNA) retained inside the BGs. The plasmid ng is converted into the copy number based on following formulae [copy number = (ng of DNA x 6.022 x $10^{23}$) / (size of plasmid in bp. x 1 x$10^9$x 660)] where $6.02214199x10^{23}$ is Avogadro's number. Size of mcDNA= 2173bp, and mpDNA= 5666bp

To find out whether the amount of mcDNA or mpDNA being quantified is totally coming from the fully form mcDNA / mpDNA, a final step of melt curve analysis at 60°C to 95°C with the interval of 10sec is applied at the end of qPCR. Different PCR products based on their size melt at different temperatures, since SYBR Green does not differentiate between the two different size DNA product, it is therefore important to verify that the PCR product being amplified through this method has same melting temperatures. During melt curve analysis the temperature of the reaction is increased by a certain factor and at certain point the DNA strand (depending on their size) is denatured, which then results in detachment of SYBR Green from double stranded DNA. This results in decrease in fluorescence signal as SYBER Green when attached to double stranded DNA molecule gives 100 times more fluorescence. The drop in fluorescence is recorded and plotted on a graph by special software provided by the manufacturer; a single narrow peak indicates the presence of single PCR product. A melt curve graph generated for mcDNA quantification is shown in (Fig.13) A single narrow peak present at same temperature can be seen which is indicative of single PCR product being amplified this could be further verified and confirmed after loading the product on 2% agarose gel. The supplementary data for the quantification of mcDNA / mpDNA related to PCR curve and standard curve generated through serial dilutions of purified mcDNA and mpDNA can be found in the Appendix. The qPCR products from two different runs was subjected to 2% agarose gel, which also confirms the presence of single amplified product at around 221bp for mcDNA (Fig.14a) and 238bp product for mpDNA (Fig.14b).

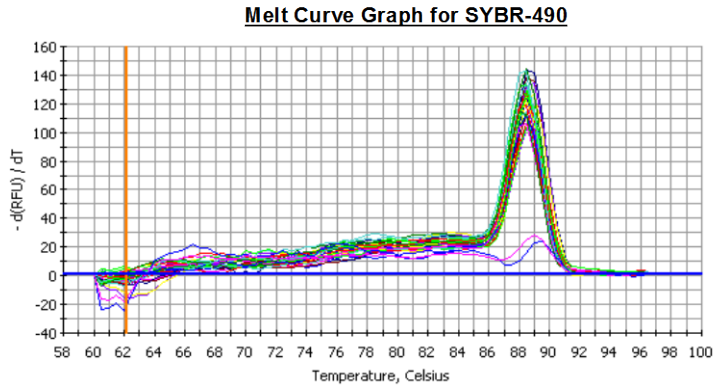


**Fig.13.** ***Melt curve Graph for SYBR-490;*** *Primer used McXF and McSR to amplify 221bp region of fully formed mcDNA. Single peak shows the specificity of PCR as different size products melt at different temperatures; -d(RFU)/dT; change in relative fluorescence unit with time.(T)*

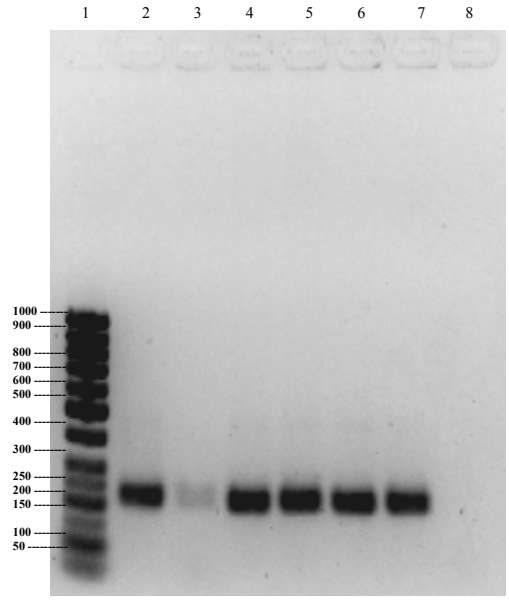


**Fig. 14a.** ***qPCR product from mcDNA quantification;*** *2% agarose gel picture of qPCR product showing amplification of 221bp mcDNA; lane 1, 100bp DNA ladder (fermentas); lane 2 and 3, mcDNA plasmid standard $10^{-2}$ $10^{-7}$; lane 4 to 7, mcDNA isolated from BG pellete collected at different time points; lane 8 netagive control.*

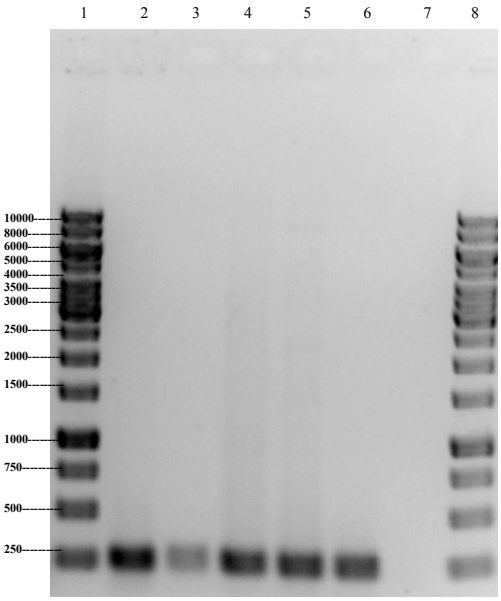


**Fig. 14b.** ***qPCR product from mpDNA quantification;*** *2% agarose gel picture of qPCR product showing amplification of 238bp mpDNA; lane 1, 1kb DNA ladder (fermentas); lane 2 and 3, mpDNA standard $10^{-2}$ $10^{-7}$; lane 4 to 6, mpDNA isolated from BG pellete collected at different time points; lane 7 netagive control; lane 8, 1kb DNA ladder (fermentas)*

This system has another advantage over the previous methods due to its ability to calculate recombination efficiency through qPCR. The primer set used for calculating the recombination efficiency is McSF and MpYR. The primer McXF present on the polyA tail and MpYR present on ParA gene is distributed among mcDNA and mpDNA respectively upon recombination (Fig.5). Thus by quantifying the amount of replicating plasmid species i.e. mpDNA and mopDNA in the BG pellet the recombination efficiency can be calculated. The plasmid p4aEYFP-C1 in an experiment showed recombination efficiency of 99.73% (Table.3).

**Table.3. Recombination efficiency (RE) of plasmid p4aEYFP-C1 through qPCR**

| Primer | $r^2$ | Slope | RE in % | mpDNA copy number/ $4.32x10^7$ Cells | mopDNA copy number/ $4.32x10^7$ Cells |
|---|---|---|---|---|---|
| MpAF MpYR | 0.984 | -3.333 | 99.73 | 2.14E+10 (133ng) | --- |
| McXF MpYR | 0.994 | -3.687 | | ---- | 5.56E+09 (47.8ng) |

Recombination efficiency calculated by formulae [E=100- (mopDNA copy / mpDNA copy]
Primer; MpAF and MpYR , primer set for quantifying midiplasmid DNA (mpDNA); McXF and MpYR, primer set used to quantify mother plasmid DNA (mopDNA); $r^2$, r square value for assessment of standard curve fit; Slope, slope for standard curve. The plasmid ng is converted into the copy number based on following formulae [copy number = (ng. of plasmid DNA x 6.022 x $10^{23}$) / (size of plasmid DNA in bp. x 1 x$10^9$x 660)] where 6.02214199x$10^{23}$ is Avogadro's number. Size of mpDNA= 5666bp, and mopDNA= 7838bp

### 3.4 Discussion

Minicircle DNA is a promising technique in DNA vaccination with broad range of applications in future clinical studies. However there has been no proper technique for its quantification. Present study is focused on the real time PCR quantification of mcDNA along with mpDNA and un-recombined mopDNA. In this study a quantification method for monitoring the recombination, immobilization and lysis event was developed by quantifying the amount of mcDNA produced, immobilized and or released after the lysis process (Fig.11a). In previous studies mcDNA was quantified through primers against LacOs sequence which is present both on fully formed mcDNA and on un-recombined mopDNA, therefore it was difficult to draw any conclusion about the source of quantified mcDNA. A densitometric analysis of linearised recombination product was necessary for calculating recombination efficiency thus making sure that the amount of mcDNA being quantified is only coming from lacOs sequence present on fully formed mcDNA and not from the mopDNA [31]. Previously used strategy for mcDNA quantification was based on assumption that only mcDNA is anchored inside the BGs where as mopDNA is released via lysis tunnel, this theory itself is debatable, as by having a closer look to mechanism of self immobilization of plasmid DNA will give us better insight about the underlying mechanism through which the LacI-L' protein is synthesized and anchored inside the bacterial membrane. This LacI-L' protein will then recognize and bind to the lacOs sequence which is present on both i) fully formed mcDNA and ii) un-recombined mopDNA. Hence it is more likely possible that a

certain amount of un-recombined mopDNA is also anchored inside the BGs via lacOs, this argument is supported by the real time PCR data for quantification of mcDNA in which it is observed that not all of the mcDNA formed due to recombination event is anchored inside the BGs, a certain amount of mcDNA is expelled via lysis tunnel (fig.12a). This could only be possible if there is no free anchoring machinery available as they are being already occupied by the lacOs present on the un-recombined mopDNA thus resulting in the release of mcDNA into the medium. Further, determining the recombination efficiency through densitometric analysis of recombination product is always a qualitative and not the quantitative. Therefore by introduction of this new quantification technique, recombination efficiency can be calculated accurately through qPCR analysis.

The reporter genes chosen for this study has advantage over the LacZ (β-gal assay) as they don't require any kind of substrates for their visualization inside the living cells [40]. The resulting increase in fluorescence signal observed by subsequent cloning of the following reporter genes (EYFP, RFP and VenusA206K) into plasmid p3a and p4a could be due to the removal of multiple cloning site (MCS) present in between the respective fluorescent genes and the polyA signals (Fig 1a and 2a). The MCS was necessary to remove before cloning the amplified PCR product containing the eukaryotic promoter (CMV) and desired reporter gene into advanced version of SIP plasmid system which has ability to recombine and anchor inside the bacterial cell wall through LacI-L' anchor protein. This removal of 51bp MCS resulted in decrement of the gap between the fluorescent proteins and polyA tails. The polyA tails has an important role in mRNA stability and its transportation within the cells [41, 42]. Therefore by removing the extra sequences in between the polyA tail and the fluorescence genes might have resulted in better mRNA stability thus leading to efficient translation of the reporter gene which results in better and higher fluorescent signals. In conclusion the cloning of reporter gene onto the improved version of SIP plasmid resulted in better and higher fluorescence signals then their origin plasmids when tested in CCL-20.2 cells.

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Chapter IV

# Improvement of Self Immobilization Plasmid used in production of minicircle DNA loaded Bacterial Ghosts

## 4.1 Introduction

Self immobilization plasmid (pSIP) contains all the essential features for its efficient immobilization inside the bacterial membrane. Use of DNA as a vaccine candidate has opened a new doors in fighting infectious diseases [1]. However their ability to elicit better immune response is entirely dependent on efficient delivery system [2] Bacterial Ghosts (BGs) are used to deliver DNA vaccine by anchoring them to the bacterial inner membrane via pSIP system[3]. In pSIP the plasmid DNA carries a tandem repeat of modified lactose operator sequences (lacOs) which is recognized by the LacI-L' fusion anchor protein which is composed of lactose repressor (LacI) and hydrophobic membrane anchor L' derived from phage MS2 [3]. The pSIP system was combined with ParA mediated recombination of mother plasmid DNA mopDNA to produce minicircle DNA (mcDNA) devoid of bacterial backbone sequences (BB) [4]. It has been shown in certain studies that transgene silencing is caused due to the covalent attachment of BB to the transcription unit [5]. Hence the mcDNA devoid of BB shows much better and gives higher level of transgene expression [6, 7].

BGs loaded with mcDNA are produced in a single one step procedure through ParA mediated recombination of mopDNA into mcDNA and mpDNA and the attachment of mcDNA inside the bacterial membrane via LacI-L' fusion protein followed by gene *E* mediated lysis of bacterial cell wall [4]. The pSIP plasmid used for production of this mcDNA carries ampicillin resistance gene. The European Medicines Agency (EMA), formerly European Agency for Evaluation of Medicinal Products (EMEA), and Food and Drug Administration of the USA (FDA) strictly prohibit the use of penicillin and other β-lactam antibiotics during vaccine production processes due to serious biosafety risks posed by these sequences [8-10]. Therefore these antibiotic resistance cassettes must be replaced by other antibiotics that are not widely used in medicinal practice. For this purpose the above mentioned regulatory agencies recommend only the use of Kanamycin or Neomycin

antibiotic resistant markers for the purpose of plasmid DNA vaccination or gene therapy as these aminoglycoside antibiotics are not widely used in medical practice [8-11].

In this study improvements has been made in previous version of SIP plasmid in order to allow cloning of homing endonuclease gene which is induced by the pBAD promoter [12] only after the ParA mediated recombination of mopDNA into mcDNA and mpDNA further more to make it available for use in vaccine production the current ampicillin antibiotic resistance gene is replaced by the kanamycin resistance which is favored by the regulatory agencies like FDA and EMA due to their less use in medical practice. This change in kanamycin resistant gene is carried out in two of the newly cloned SIP plasmids i.e. p3a and p4a. The plasmid p4a has ability to recombine into mcDNA carrying only the lacOs and the MCS for easy cloning of reporter gene in it and the mpDNA carrying BB and antibiotic resistance gene. The plasmid p3a lack recombination due to absence of resolution site 1.

## 4.2 Material and methods.

### 4.2.1 Bacterial strains, plasmids and growth conditions

#### 4.2.1.1 Bacterial strains

*E. coli K12 C2988J (NEB 5-α competent cell) fhuA2 Δ (argF-lacZ) U169 phoA glnV44 ϕ80Δ (LacZ) M15 gryA96 recA1 endA1 thi-1 hsdR17)* (New England Biolabs, Germany)

#### 4.2.1.2 Growth conditions

The bacterial cultivation was carried out in Luria-Bertani (LB) medium [13] with ampicillin (100µg/ml) and kanamycin (50µg/ml) when needed. The LB plates and medium used for cloning and growth of plasmid p4a was supplemented with glucose at final concentration of 2% in order to prevent premature recombination.

### 4.2.1.3 Plasmids

Plasmid pSIPHCNparA [4], plasmid pBHR1 [14], plasmid pSIPHCNparA-res1 (this work), p2a (this work), p3a (this work), p4a (this work), p3aKan (this work) and p4akan( this work).

## 4.3 Results and discussion

### 4.3.1.1 Construction of plasmid p3a

To construct Plasmid p3a two intermediate plasmid constructions (pSIPHCNparA-res1, and p2a) was carried out. For easy handling, plasmid pSIPHCNparA [4] (Fig.1a) was digested with *HindIII* (Fermentas) double cut and was re-ligated to remove the resolution site 1 (res1 140bp). The resulting plasmid was named pSIPHCNparA-res1 (6268bp) (Fig.1a) lacking recombination activity. Suitable restriction sites or multiple cloning sites MCS was introduced into this plasmid pSIPHCNparA-res1 by digesting it with *Nsi*I (Fermentas) and the 60bp MCS (Synthesized) was inserted into this site the resulting annealed plasmid is then called p2a (6128bp) (Fig.1b).

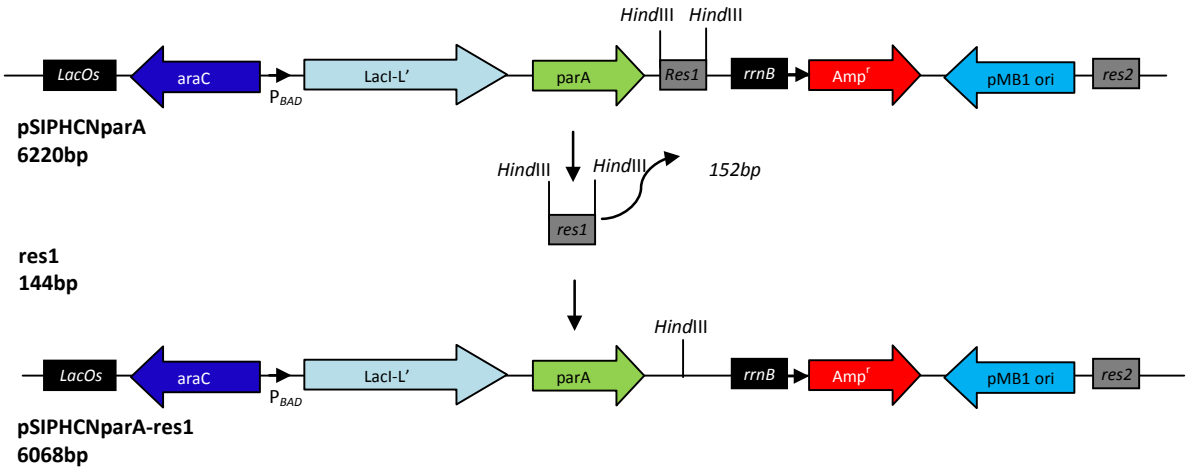


**Fig.1a.** ***Cloning strategy of plasmid pSIPHCNparA-res1;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

This was done by mixing and incubating two primers synthesized for MCS (MCS1 and MCS2) and by introducing this annealed MCS into respective restriction site present on

plasmid pSIPHCNparA-res1 using T4 DNA ligase (New England BioLabs, Germany). In order to allow the easy cloning of lethal gene (e.g. *I-Tev*II homing endonuclease that is needed for another study involving plasmid p3a) the expression cassette (LacI-L'/ParA) should be inverted for tight repression and prevention of premature expression of cloned gene. *i.e.* before recombination. For this purpose plasmid p2a was digested with *Hind*III (Fermentas) which cuts the plasmid at two different locations and the plasmid was re-ligated using T4 DNA ligase (New England BioLabs, Germany) to get plasmid p3a (6128bp) (Fig. 1c).

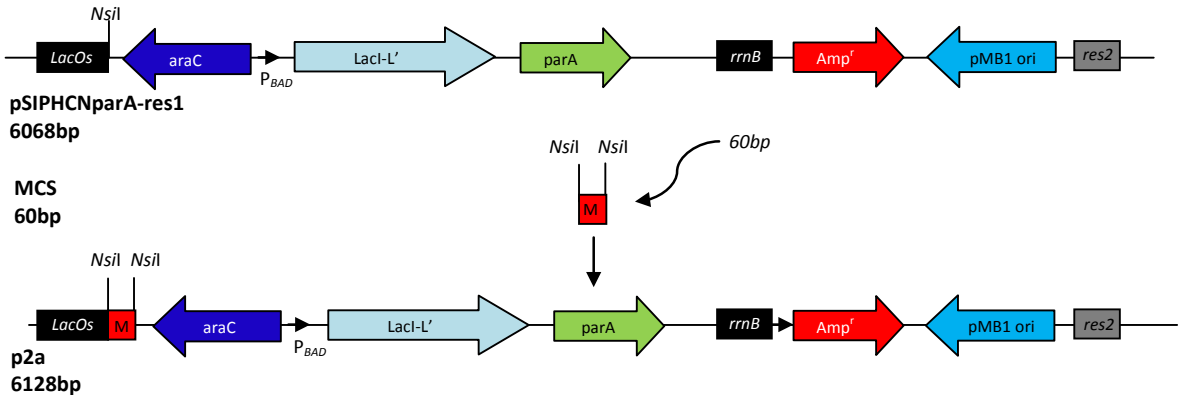


**Fig. 1b*: Cloning strategy of plasmid p2a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
MCS1: 5'-ATGCATTAATTAACTAGTGAGCTCACGTGCGGCCGCCCGGGTACCTGCAGTTATAAGCTTATGCAT-3'
MCS2: 5'-TACGTAATTAATTGATCAGTCGAGTGCACGCCGGCGGGCCCATGGACGTCAATATTCGAATACGTA-3'

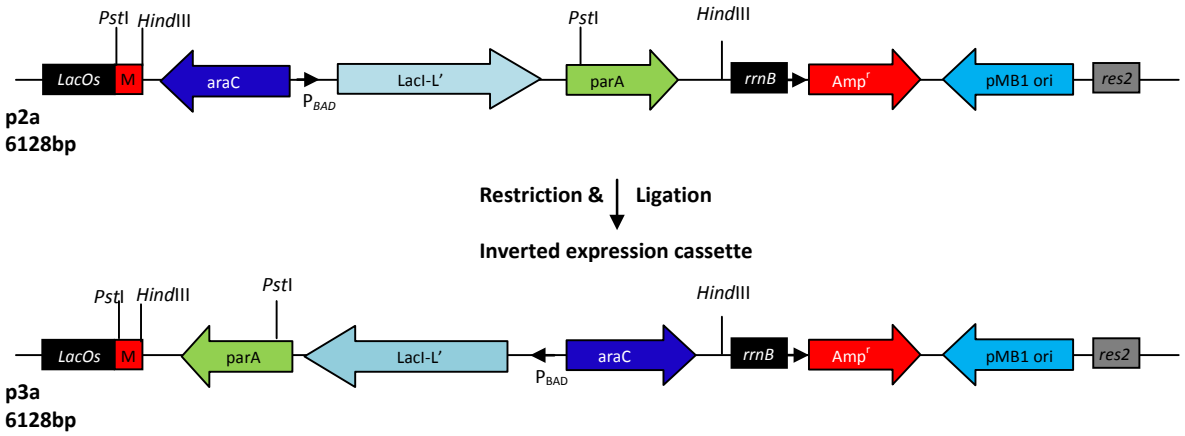


**Fig. 1c*: Cloning strategy of plasmid p3a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*

### 4.3.1.2 Construction of plasmid p3aKan

To replace the antibiotic resistance gene of plasmid p3a from ampicillin to kanamycin we amplified 934bp kanamycin fragment from the plasmid pBHR1(5300bp) [14] using the primers listed in (Table.1) and inserted into the *Ahd*I restriction site of linearised p3a plasmid, resulting in plasmid p3aKan (7062bp) (Fig.1d) The Ampicillin gene of plasmid p3a is destroyed by *Ahd*I restriction digestion. The polymerase chain reaction (PCR) reaction was carried out as follows. 25µl of total reaction containing 0.25µl of (50 pmol/µl) primers each (Microsynth AG), 2.5ul of (2mM) dNTP's (Fermentas), 2.5ul of (10x) DreamTaq buffer (Fermentas), 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U/ul).

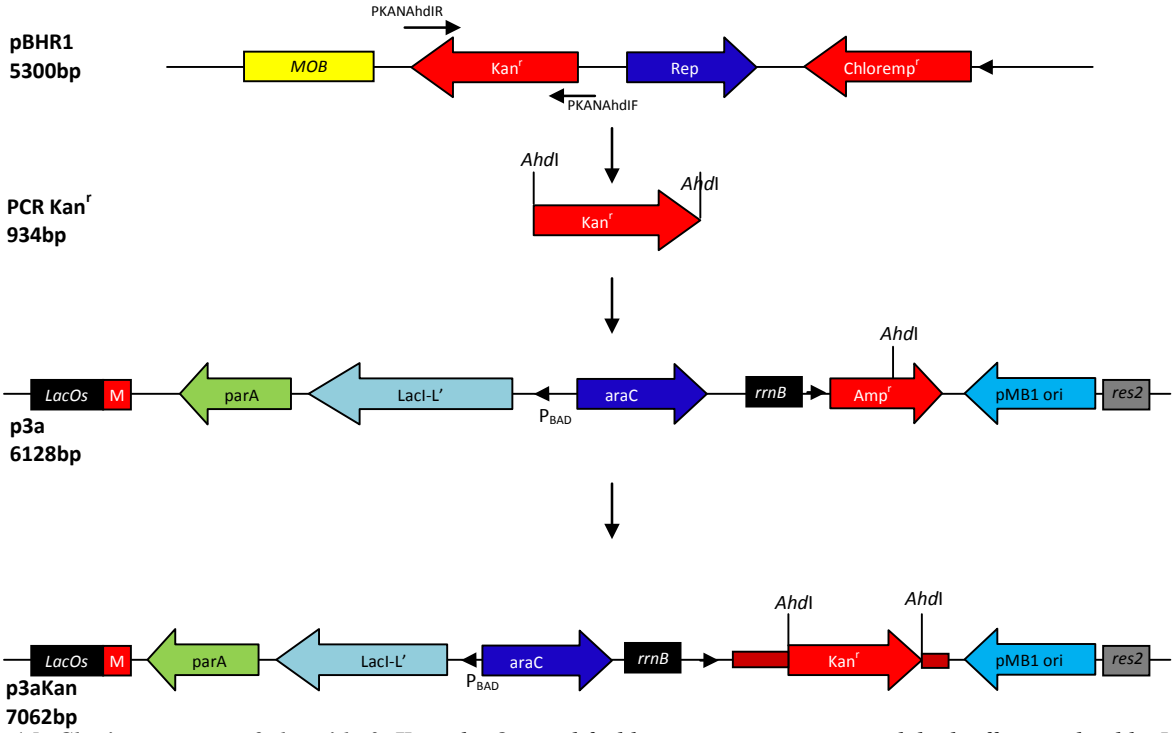


**Fig.1d.** ***Cloning strategy of plasmid p3aKan;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; Kan$^r$, kanamycin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.MOB; mobilization sequence; rep; origin of replication; Chloramp$^r$, chloramphenicol resistance gene*

The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95°C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95°C for 30sec,

60°C for 30sec and 72°C for 1min and final elongation of 72°C for 10min. The PCR product was analyzed on 2% agarose gel and subsequently digested with *Ahd*I (Fermentas) both the Vector and insert were purified using PureLink™ PCR purification kit (invitrogen) following manufacturer's instructions. The vector fragment was dephosphorelated with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) before purification step to prevent re-ligation and subsequently cloned into the corresponding *Ahd*I site in plasmid p3a to get plasmid p3aKan.

The ligation product from the cloning procedure of plasmid p3akan was used to transform the *E.coli* C2988J mops competent cells according to the standard molecular biological techniques by Sambrook *et.al.* [15]. About 20 bacterial colonies were picked and inoculated into 5ml Luria-Bertani (LB) medium [13] supplemented with kanamycin (50µg/ml). The plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). The samples were digested with restriction enzymes (purchased form Fermentas) listed in plasmid collection map and were loaded on 1% agarose gel (Roth™) stained using gel red nucleic acid gel stain (GelRed™ Biotium # 41003) and analyzed under UV light in a ChemiDOC™ machine (BioRad laboratories) for analyzing the right clones. A restriction digest of positive clone of plasmid p3aKan is shown in (Fig.1e).

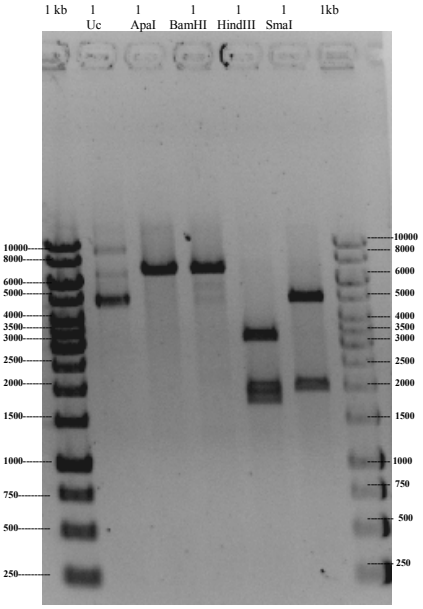


**Fig.1e.** ***Restriction analysis of plasmid p3aKan;*** *1% agarose gel picture showing positive clone of plasmid p3aKan; lane1, GeneRuler™ 1kb DNA ladder (Fermentas); lane2, uc,7062bp; lane3, Apa*I *7062bp; lane4,BamH*I *7062bp; lane5,Hind*III *1799 / 1973/ 3290bp; lane6, Sma*I *2026/5036bp; lane7, GeneRuler™ 1kb DNA ladder (Fermentas).*

| Table.1. Primers list | | | |
|---|---|---|---|
| Template | Amplified region | Primer | Fragment size |
| pBHR1 | Kanamycin resistance cassette | PKanAhdIF 5'-TTA GCA *GAC GG'G GAG TC*G CCA CGT TGT GTC T -3' | 934bp |
| | | PKanAhdIR 5'- CAC CAG *GAC GG'G GAG TC*T TAG AAA AAC TCA T -3' | |
| pSIPHCNparA | Resolution site 1 | 5'res1K 5'– CAG CAG *GGT ACC* CCT TGG TCA AAT TGG GTA TAC C –3' | 140bp |
| | | 3res1P 5' – CTG CTG *TTA TAA* GCA CAT ATG TGG GCG TGAG – 3' | |
| Primers designed using web based primer3 software using default settings | | | |

#### 4.3.2.1 Construction of plasmid p4a

For construction of plasmid p4a the plasmid p3a was used as a backbone, the resolution site 1 was introduced into the plasmid p3a in order to get the final working plasmid p4a (which has the ability to recombine). For this purpose the 140bp res1 site was amplified through PCR using plasmid pSIPHCNparA [4] as template and primers 5'res1K and 3res1P (synthesized by Microsynth AG, Switzerland) with *Kpn*I and *Psi*I restriction sites *Italicized* sequence in primer (Table.1)

The PCR reaction with final volume of 25µl was carried out using 0.25µl of (50 pmol/µl) primers each, 2.5ul of (2mM) dNTP's(Fermentas), 2.5ul of (10x) DreamTaq polymerase (Fermentas) buffer, 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U/ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95$^{o}$C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95$^{o}$C for 30sec, 60$^{o}$C for 30sec and 72$^{o}$C for 1min and final elongation of 72$^{o}$C for 10 min. The PCR product was analyzed on 2% agarose gel to confirm the amplification. The PCR product was digested with *Kpn*I and *Psi*I (Fermentas) and subsequently cloned into the corresponding sites in plasmid p3a to get the vector p4a (6263bp) (Fig.2a).

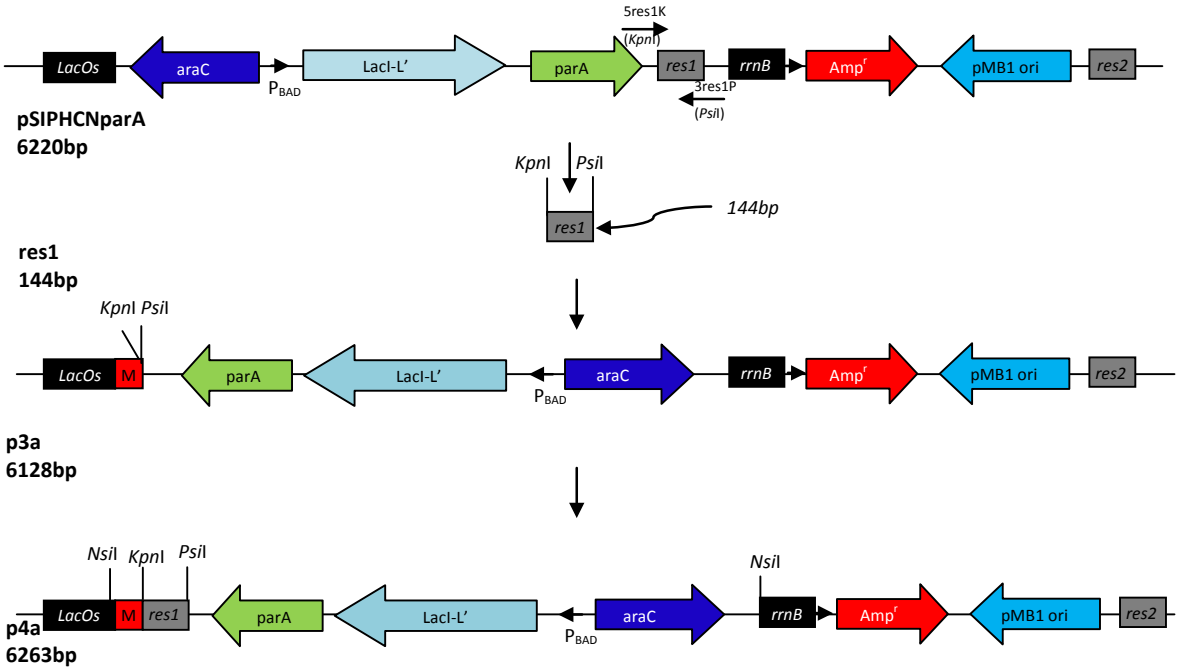


**Fig.2a.** ***Cloning strategy of plasmid p4a;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; pMB1, origin of replication derived from pMB1 plasmid.*
*5'res1K 5'– CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3'*
*3res1P 5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3*

### 4.3.2.2 Construction of plasmid p4aKan

For construction of plasmid p4aKan (Fig.3a) the resolution site1 was introduced into the plasmid p3a. For this purpose the 140bp res1 site was amplified through PCR using plasmid pSIPHCNparA [4] as template and primers 5'res1K and 3res1P (synthesized by Microsynth AG, Switzerland) (Table.1). The PCR reaction with final volume of 25µl was carried out using 0.25µl of (50 pmol/µl) primers each, 2.5ul of (2mM) dNTP's (Fermentas), 2.5ul of (10x) DreamTaq buffer (Fermentas), 1ul of template DNA, and 0.25ul of DreamTaq polymerase (Fermentas) at final conc. of (0.05U/ul). The PCR condition was optimized based on the melting temperature (Tm) of the primers, using iCycler iQ Real-Time PCR detection system from Bio-Rad Inc. initially 95$^o$C for 3min, as pre de-naturating temperature was used followed by 30 cycles of 95$^o$C for 30sec, 60$^o$C for 30sec and 72$^o$C for 1min and final elongation of 72$^o$C for 10min. The amplification of PCR product was checked on 2% agarose gel and subsequently digested with *Kpn*I and *Psi*I (Fermentas) and subsequently cloned into the corresponding sites in plasmid p3aKan resulting in vector p4aKan (7197bp). A positive clone of plasmid p4aKan is shown in (Fig.3b). The plasmid p4aKan is under control of

pBAD promoter and has ability to recombine into mcDNA and mpDNA upon addition of L-(+)-arabinose.

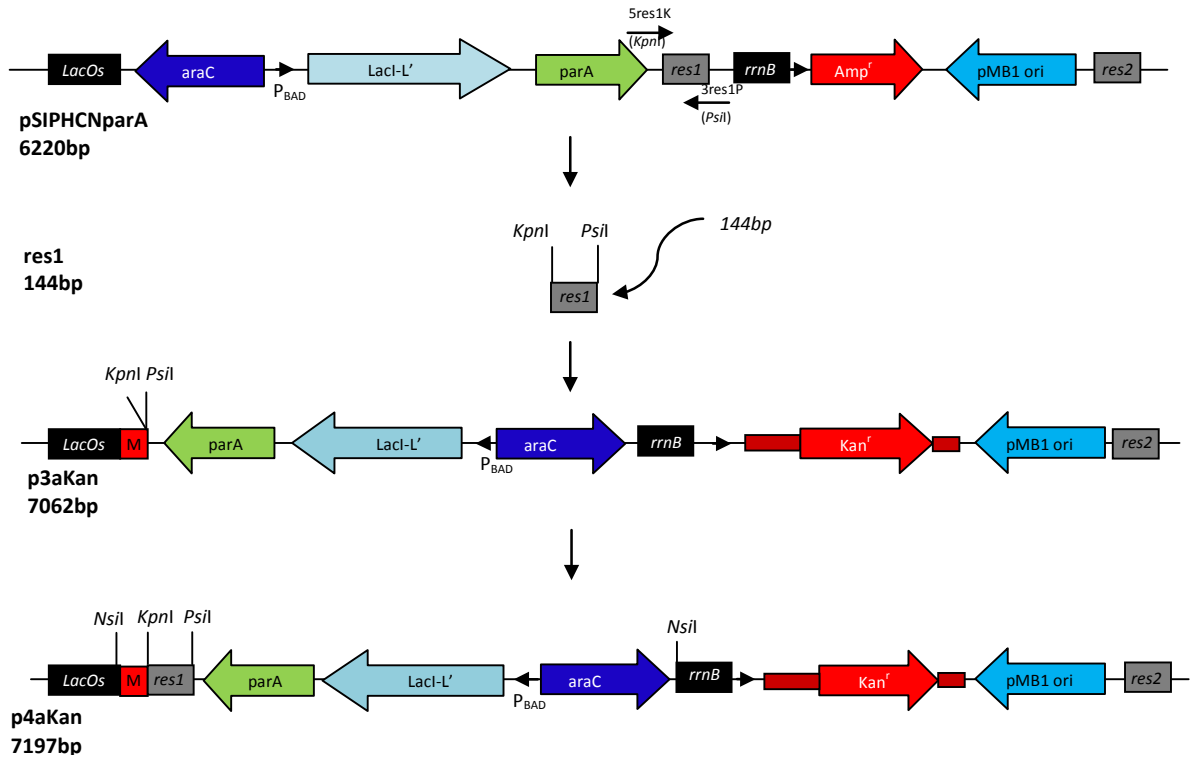


**Fig.3a.** ***Cloning strategy of plasmid p4aKan;*** *lacOs, modified lac operator sequence with high affinity to bind lacI; M, Multiple cloning site; res1, resolution site 1; res2, resolution site 2; parA, ParA resolvase gene; lacI-L', fusion protein of lacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; $P_{BAD}$, arabinose inducible promoter; araC, repressor/inducer of $P_{BAD}$ promoter; rrnB, transcriptional terminator sequence of 5s ribosomal gene; Amp$^r$, Ampicillin resistance gene; Kan$^r$, kanamycin resistance gene pMB1, origin of replication derived from pMB1 plasmid.*
*5'res1K 5'– CAG CAG GGT ACC CCT TGG TCA AAT TGG GTA TAC C –3'*
*3res1P 5' – CTG CTG TTA TAA GCA CAT ATG TGG GCG TGAG – 3*

The ligation product from the cloning procedure of plasmid p4akan was used to transform the *E.coli* C2988J mops competent cells according to the standard molecular biological techniques by Sambrook *et.al.* [15]. About 15 bacterial colonies were picked and inoculated into 5ml Luria-Bertani (LB) medium [13] supplemented with kanamycin (50µg/ml). The plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). The samples were digested with restriction enzymes (purchased form Fermentas) listed in plasmid collection map and were loaded on 1% agarose gel (Roth$^{TM}$) stained using gel red nucleic acid gel stain (GelRed$^{TM}$ Biotium # 41003) and analyzed under UV light in a ChemiDOC$^{TM}$ machine (BioRad laboratories) for analyzing the right clones. A restriction digest of positive clone of plasmid p4aKan is shown in (Fig.3b).

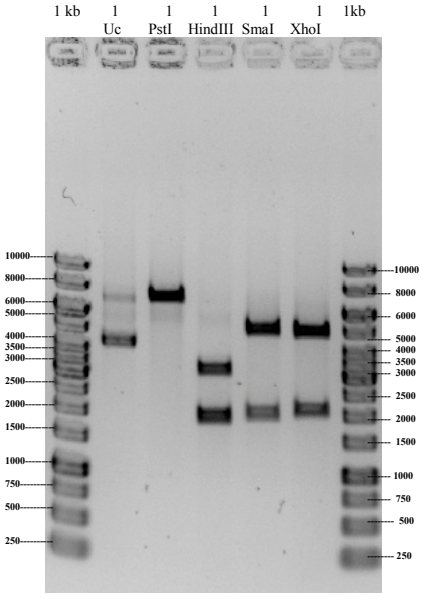


**Fig.3b.** ***Restriction analysis of Plasmid p4aKan;*** *1% agarose gel picture showing successful cloning of res1 site in plasmid p3aKan ; lane1, GeneRuler™ 1kb DNA ladder (Fermentas); lane2, uc,7197bp; lane3, Pst*I *7197bp; lane4,Hind*III 1934/1974/3290*bp; lane5, Sma*I *2026/5171bp; lane6, Xho*I *48/2097/5060bp; lane7, GeneRuler™ 1kb DNA ladder (Fermentas).*

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Publication II

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# Bacterial Ghosts as carriers of protein subunit and DNA encoded antigens for vaccine applications

Abbas MUHAMMAD [1], Jonathan CHAMPEIMONT [1], Ulrike Beate MAYR [2], Werner LUBITZ [1,2], Pavol KUDELA[2,3,*]

[1] Centre for Molecular Biology, University of Vienna, A-1030 Vienna, Austria

[2] BIRD-C GmbH&CoKG, Hauptstrasse 88, A-3420 Kritzendorf, Austria

[3] Cancer Research Institute, Slovak Academy of Sciences, SK-83391 Bratislava, Slovakia

[*]Corresponding author:

Pavol KUDELA, PhD.

BIRD-C GmbH&CoKG — Tel.: +43-664-928-7851

Hauptstrasse 88 — Fax.: +43-1-2243-28514

A-3420 KRITZENDORF — e-mail: pavol.kudela@bird-c.at

Austria

**Running title:** BG-Delivery System for Human and Veterinary Medicine

**Keywords:** Bacterial Ghosts; Vaccine; Antigen delivery system; Natural adjuvant.

**Abstract**

Bacterial Ghosts (BGs) represent vaccine delivery systems gifted with outstanding natural adjuvant properties. BGs are empty cell envelopes of Gram-negative bacteria lacking cytoplasmic content yet retaining unaltered all morphological and structural features of their living counterparts. BGs intact surface make-up is easily recognized by professional antigen-presenting cells through pattern recognition receptors making them ideal for mucosal administration through oral, ocular, intranasal or aerogenic routes, which represent the most desirable ways of application in advanced vaccine use. BGs have been designed to be used as carrier of active substances and foreign antigens (protein and/or DNA) for vaccine development. This review highlights the salient features of the BGs versatile multipurpose vaccine platform for application in a wide range of human and veterinary medicines.

**Abbreviations**

Ag, antigen; APCs, antigen-presenting cells; BB, bacterial backbone; BGs, Bacterial Ghosts; BPL, β-propiolactone; CMV, cytomegalovirus; DCs, dendritic cells; EHEC, enterohemorragic *Escherichia coli*; EMA, European Medicines Agency; FDA, Food and Drug Administration; GnRH, gonadotrophin-releasing hormone; HA, heamagglutatnin; HBV, hepatitis B virus; hCG, human chorionic gonadotrophin; HIV, human immunodeficiency virus; IE, immediately early enhancer; IM, inner membrane; LPS, Lipopolysaccharide; MBP, maltose-binding protein; mcDNA, minicircle DNA; mpDNA, miniplasmid DNA; NK cells, natural killer cells; NTHi, nontypable Haemophilus influenzae; OM, outer membrane; ompA, outer membrane protein A; PAMPs, pathogen-associated molecular patterns; pSIP, self-immobilizing plasmids; RITARD, reversible intestinal tie adult rabbit diarrhea; SNUC, *Staphylococcus aureus* nuclease A; TLR, toll-like receptor; TCP, Toxin-coregulated pilus; ZP, zona pellucida.

**Key issues**

- The Bacterial Ghost platform technology represents a novel, safe, cost effective, progressive and versatile multipurpose approach in the development of safe and potent vaccines in the prophylaxis of a variety of infectious diseases.
- Successful delivery of Ags from various pathogens to target cells requires the adequate form and suitable compartments of the selected delivery system, and should induce strong humoral and cellular immune responses, and long protections against the disease caused by pathogen or change the behavior of targeted cells.
- The selected delivery system possessing excellent adjuvant properties should not represent any potential hazard for horizontal gene transfer and must demonstrate a suitable safety profile and stability at room temperature permitting its easy storage and handling in less developed countries.
- BGs can be produced very efficiently by fermentation in disposable or conventional steel fermenters of various sizes in research laboratories or in large GMP units of pharmaceutical industry. The initial engineered working stocks of target bacteria can easily generate large quantities of a BGs vaccine in a short period of time at low costs.
- Optimization and improvement of the selected prospective model type of BGs would help to progress the development of microbial-mediated disease vaccine and their application in future clinical trials.

The word vaccine (Latin word *vacca* means cow) is derived from the work of Edward Jenner who inoculated cowpox in humans consequently protecting them against smallpox [1-2], hence providing one of the first clues pointing to the relationship between human and veterinary infectious disease sciences. In 1960 Ito et al. discovered the property of naked DNA to transfect mammalian cells *in vivo* [3]. Later on, this concept was further implemented by Wolf et al. when he and his colleagues inoculated plasmid DNA carrying a reporter gene into the muscle of mice which resulted in *in vivo* expression of the encoded gene [4-5]. Since then the use of naked DNA as a vaccine has been intensively studied and a variety of such vaccines using naked DNA have undergone clinical trials in both human and veterinary practices [6-9]. Although the use of DNA vaccines as intramuscular injection is a quite simple procedure, the mechanism responsible for the induction of a specific immune response was proven to be multifaceted as most of the cells transfected by this method are non-effective in the antigen (Ag) presentation and priming of naïve immune cells [4,10-11]. These facts provide a need for the development of an efficient DNA delivery system capable to target professional antigen-presenting cells (APCs), e.g. macrophages and dendritic cells (DCs), and stimulate strong and efficient Ag-specific immune responses. Among commonly used viral and non-viral DNA delivery systems the non-viral DNA delivery systems are more promising due to their simplicity and higher safety regardless their reduced transfection efficiencies [12-13].

The most commonly used veterinary vaccines are killed microorganisms (bacteria) inactivated either by heat, irradiation or chemical treatment. Unfortunately, during this "inactivation" process most of the essential structural components of the bacterial cell wall are denatured resulting in impaired function and non-efficient immune responses [14-20].

Recently several synthetic systems serving as adjuvants capable to increase the poor immunogenicity of defined highly purified Ags when using subunit vaccines have been intensively studied. Membrane vesicles – liposomes– made of one (unilamellar) or more (multilamellar) phospholipid membranes containing both hydrophobic and hydrophilic domains represent interesting delivery systems capable after specific modifications to efficiently stimulate specific immune responses [21]. The hydrophilic core of liposomes can protect Ags or drugs from degradation by environmental impacts after particular administration before reaching the final destination [22]. Moreover, liposomes allow using lower concentrations of highly toxic or poorly soluble compounds bringing them directly to specific target cells. Only few anticancer drug loaded liposomes were used in veterinary

medicine [23-25]. The cationic surface of liposomes and its modulation with immunostimulators including pathogen-associated molecular patterns (PAMPs) such as LPS, monophosphoryl lipid A, flagellin and others enhance the recognition and internalization by APCs hence having a positive impact on their maturation leading to effective stimulation of Ag-specific immune responses [21,26-30]. However, administration of novel adjuvants or vaccines comprising non-biolological combinations of various ligands for immunomodulatory receptors presented on the surface of APCs or other target cells should be considered very carefully, because inappropriately selected combinations might lead to unspecific immunostimulatory signals and unpredicted adverse effects. These facts will lead to more thorough safety studies required by regulatory agencies and have a negative impact on production costs and the final price of the product [31].

The Bacterial Ghost system represents a novel and progressive approach in the development of safe and potent vaccines in the prophylaxis of a variety of infectious diseases [14,32-33]. Bacterial Ghosts (BGs) are produced by controlled expression of cloned gene *E* of bacteriophage ΦX174 [32-36], resulting in the formation of a trans-membrane tunnel structure spanning the whole cell envelope, through which the entire cytoplasmic content is expelled due to the change in osmotic pressures between the cell interior and the culture medium [37]. The empty inner space of BGs can be filled with drugs, proteins, DNA, enzymes and other compounds. Additionally, BGs can be closed or sealed with membrane vesicles if desired [38-39]. The main advantage of BGs compared to other non-living vaccines is the fact that all surface structural components of the envelope are non-denatured and remain intact [40]. The induced lysis process does not harm the essential structural components of the bacteria giving raise to immunologically active particles capable to stimulate the host immune system and deliver specific Ag to professional APCs or active substances to the target cells [41]. Production of BGs is an efficient, stable, and safe process resulting in freeze dried vaccine preparations which are stable at ambient temperatures for many months [42]. In addition to multiple immunization ways used for successful administration of BGs, e.g. intradermal, intramuscular, intravenous, subcutaneous, intraperitoneal, oral, aerosol, intragastric etc., prepared BG formulations can be also effectively administered through other mucosal routes [43-56]. This review will highlight the potential of BGs to be used as carriers of protein subunit and DNA vaccines, their use as a combination vaccine, and summarize results from applications in veterinary vaccine trials, and preclinical and animal studies for human vaccine candidates.

**Bacterial Ghost System**

BGs are empty cell envelopes of Gram-negative bacteria produced by controlled expression of lysis gene *E*. Gene *E* codes for 91 amino acids and exerts its lytic function in Gram-negative bacteria by fusion of the inner and outer cell membranes forming a specific transmembrane tunnel structure, through which all the cytoplasmic content is expelled leaving a bacterial envelope called Bacterial Ghost devoid of nucleic acids, ribosomes and other intracellular constituents [57-61]. Induced E-specific transmembrane lysis tunnels are of different size (40-200 nm) and are more abundant about 2/3 in the potential zone of cell division in the middle of bacteria than at the polar sites [40,58,62-64]. The inner membrane (IM) and outer membrane (OM) structures of BGs remains intact during the lysis process [37,40,58]. Electron microscopy studies [37,58] and enzymatic studies [40,65] clearly showed a sealed periplasmic space at the border of the lysis tunnel. It is believed that protein E induces occurrence of lysophosphatidylethanol-amine in the host cell membrane, which most probably facilitates the fusion of IM and OM [64]. E-mediated lysis strictly depends on several bacterial host factors, e.g. active growth and cell division. Bacteria entering the stationary phase are phenotypically resistant to this activity [66]. Moreover, the efficient production of BGs also significantly depends on pH and osmotic pressure of the medium [42]. Detailed overview of various factors affecting the E-mediated lysis is described in Table 1.

The E-specific lysis process in growing bacteria includes integration of protein E into the IM, followed by its conformational change, which leads to the fusion of IM with OM and sealing of the periplasmic space. Transmembrane tunnel structures are build-up by the assembly of protein E into multimers at the potential cell division sites [67]. The mechanism of the conformational change is most probably due to a cis-trans isomerization of the proline residue (P21) within the first membrane-embedded α-helix of protein E [68]. The local fusion of IM and OM is completed by the transfer of the C-terminal domain of protein E towards the surface of the OM of the bacterium [67].

**Table 1. Bacterial cell factors affecting E-mediated lysis.**

| Cellular conditions/ Mutant genes | Inhibition of lysis | Retardation of lysis | Ref. |
|---|---|---|---|
| Stationary growth | Yes | No | [66] |
| Slow growth rates | No | Yes | [66] |
| Low proton motive forces (pmf) | Yes | No | [65] |
| Penicillin binding Protein mrcA (pbp1a) | No | No | [58] |
| mrcB (pbp1b) | No | No | [58] |
| pbpA (pbp2) | No | No | [58] |
| pbpB (pbp3) | No | No | [58] |
| dacB (pbp4) | No | No | [58] |
| mepA, mepB | No | Yes | [58] |
| lytA | Yes | No | [132] |
| envC + rle | Yes | No | [133] |
| envC | No | Yes | [133] |
| slyD | Yes | No | [134] |
| pxlA | Yes | No | [135] |
| ftsZ | Yes | No | [136] |
| ftsA12 | Yes | No | [136] |
| fabB, fade | No | Yes | [133] |
| wee | No | Yes | [135] |
| ftsA3 | No | No | [136] |
| ftsQ | No | No | [136] |
| relA | No | No | [137] |
| pldA1 | No | No | [64] |
| gpK | No | No | [138] |
| $MraY_{F288L}$ | Yes | No | [139] |
| BsMraY | Yes | No | [139] |

## Mechanism and expression of the lysis gene *E*

Expression of the lysis gene *E* in the host bacterium has to be under tight genetic control. Leakage in the expression control of lysis gene *E* during the BGs formation process could be lethal for the host bacterium. Bearing this in mind the expression of cloned gene *E* can be regulated by several control promoter-repressor systems including thermosensitive *λpL/pR-cI*857, the chemically inducible *lac* PO-*lacI*q, the arabinose or the TOL promoter-repressor system [33,69-70]. To further extend the heat stability of the *λpR* promoter/*cI857* repressor system Jechlinger et al. in 1999 identified and described a single point mutation in the OR2 operator region of the rightward *λpR* promoter, which enhanced the temperature stability of the *λpR/cI*857 gene expression system. This discovered specific mutation ($\lambda pR_{mut}$) strictly repress the expression of gene *E* at temperatures lower than +37°C, but allows the E lysis at higher temperatures between +38°C and +42°C [57].

Furthermore, a cold sensitive method for the production of BGs by lowering the growth temperature of bacteria down to +30°C or lower was developed. In this system the expression of lysis gene *E* is kept under the expressional control of either the *lac* PO-*lacI* or the phage 434 *cI*/pR expression system. Both repressor genes *lacI* and the phage 434 gene *cI* are regulated by transcriptional control of the rightward *λpR* promoter and the *cI857* temperature-sensitive repressor. Expression of repressors (*lacI* /*cI*) is inhibited at temperatures lower than +30°C and lack of the repressors leads to the expression of gene *E* and bacterial lysis at low temperatures. Besides carrying the repressor system on the E-mediated lysis plasmid, the corresponding repressor system preloaded in a bacterium, expressed either chromosomally or encoded on an extra plasmid, is essential to moderate the highly lethal effects of the gene *E* product [71].

**Production of BGs**

BGs can be produced by controlled expression of the lysis gene *E* in a wide range of non-pathogenic, pathogenic and probiotic Gram-negative bacteria, e.g. *Escherichia coli* K12 strains, *Actinobacillus pleuropnemoniae* (App)*, Bordetella bronchiseptica, Edwardsiella tarda*, *enterohemorrhagic Escherichia coli* (EHEC)*, enterotoxigenic Escherichia coli* (ETEC)*, Francisella tularensis LVS, Helicobacter pylori, Klebsiella pneumoniae*, *Mannheimia (Pasteurella) haemolytica*, *Neisseria meningitidis*, *Pasteurella multocida*, *Pectobacterium cypripedii*, *Ralstonia eutropha*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella flexneri*, *Vibrio cholera*, *Vibrio anguillarum* and probiotic *Escherichia coli* Nissle 1917 [14,62,72]. This demonstrates the large ability of the BG system for selection of suitable bacterial candidates in vaccine development for veterinary and human applications. Determinations of the number of total and reproductive bacteria present during the time course of growth and lysis process plus optical densities of the cultures represent standard microbiological procedures used at the time of BGs production [14].

Efficiency of the E-mediated lysis process, and quantification of generated BGs and non-lysed viable bacteria are determined by flow cytometry assays using a specific dye, which is sensitive to the changes of discriminatory power of membrane potential and stains only cells that have lost membrane potential (BGs or dead bacteria). Number of fermentation studies related to the controlled expression of the lysis gene *E* led to the development and determination of standardized conditions for the BGs generation of at least 99.9% of the total

number of bacteria in the culture. Remaining non-lysed, but reproductive bacterial cells are inactivated by β-propiolactone (BPL) to avoid the presence of any living cell already before lyophilization. BPL is chemical agent alkylating with nucleic acid mostly guanine and widely used for inactivation of viruses and for sterilization of vaccines, human tissue implants and plasma [73]. Moreover, an alternative process of nucleic acid degradation during the BGs production can be used to eliminate any danger related to nucleic acids such as horizontal gene transfer of either pathogenic or antibiotic resistance genes. To avoid any of these hazards the *Staphylococcus aureus* nuclease A (SNUC) can be expressed along with the lysis gene *E*. SNUC completely degrades the host DNA and any other nucleic acid into fragments no longer than 100 base pairs [74]. The combination of BGs formation and SNUC expression has been performed in consistency study of BGs production from *S. flexneri* with results showing the values of presented residual chromosomal or plasmid DNA below the detection limit of real time PCR [75]. In order to obtain dry-powdered BGs ready for further applications *in vivo* or *ex vivo*, produced BGs are intensively washed and freeze-dried. The whole BGs production process is kept under sterile conditions.The detailed BG production process is described in a recent review of Langemann et al. [42].

**BGs-vaccine candidates in human and veterinary medicine**

The BG system represents a novel non-living highly efficient Ag and drug delivery platform as an alternative to viral and bacterial methods applied in current vaccine development. The safety profile of DNA delivery systems applicable and effectual in human and veterinary medicines represents one of the biggest demands in the development of new DNA carrier/delivery systems. Our recent studies confirmed no cytotoxic and genotoxic impacts of BGs on the viability and metabolic activity of a wide range of tested cells including macrophages [12], dendritic cells [43,76], tumor cells [77-78], endothelial cells [79] and epithelial cells [80]. It has been shown that BGs with their intact surface structures are efficiently recognized and phagocytosed by professional APCs through various surface receptors, e.g. complement receptors and Toll-like receptors [81]. Internalization of BGs derived from *A. pleuropneumoniae* by porcine APCs led to enhanced expression of antigen-presenting molecules on the surface of APCs and significantly increased the capacity of APCs to stimulate proliferation of T cells [82]. Moreover, futher studies using DCs as model of the

most professional APCs revealed that their phagocytic activity and uptake of BGs depend on the bacterial strain used for the production of BGs [76].

**BGs-carrier of subunit proteins**

The recombinant DNA technology has been used either to anchor foreign protein Ags to the cell membrane complex of bacteria used for BGs generation or to load foreign Ags inside the BG envelopes for the use of BGs as carriers of subunit vaccines [62,83-85]. Selected Ags may be presented on the surface of BGs either via fusion with outer membrane proteins [50,86], or anchored to the IM by its fusion to N-, C- ,or N- and C- terminal membrane anchors [33]. It has been shown that these anchors do not affect the proper folding of attached Ags, e.g. β-galactosidase and polyhydroxybutyrate-synthetase [87], while their self assembly and clustering can be further manipulated [38]. This particular procedure allows the attachment of Ag to the IM keeping the structure of conformational epitopes intact and the enzymatic activities fully active [38]. Moreover, loading of target Ags into the periplasmic space can prevent sudden degradation of the Ag due to the external environmental factors or lyophilization process during BGs production. This gel resembling area of the periplasmic space is rich in membrane derived oligosaccharides [88] and is tightly sealed by the fusion of IM and OM during the lysis process [37,62]. Target Ags alone or incorporated into SbsA or SbsB S-layer fusion proteins are exported to the periplasmic space by their fusion to the maltose binding (MalE) protein [54,89-91].

Furthermore, the property of S-layer protein matrices SbsA (hexagonally ordered) and SbsB (obliquely ordered) which are forming sheet like self-assembly structures can be also explored as carriers of foreign Ags in the inner lumen of BGs. These protein structures are not expelled with the cytoplasm to the external environment during E-mediated lysis and remain in the empty BGs [90]. The p6 self-assembly lattice of S-layer protein SbsA showed higher capacity to accept foreign Ags compared to p2 lattice of SbsB due to its capacity to tolerate larger inserts e.g. Omp26 (NTHi) [92-93]. Recombinant BGs carrying the fusion protein made of Zona pellucida 3 (ZP3), SbsA and maltose-binding protein (MBP), which was expressed and transported to the periplasmic space, were used to deliver the target Ag as an immunocontraceptive vaccine in possums [94]. A schematic picture of miscellaneous possibilities for an emplacement of target Ags within the BG envelope complex is depicted in FIGURE 1.

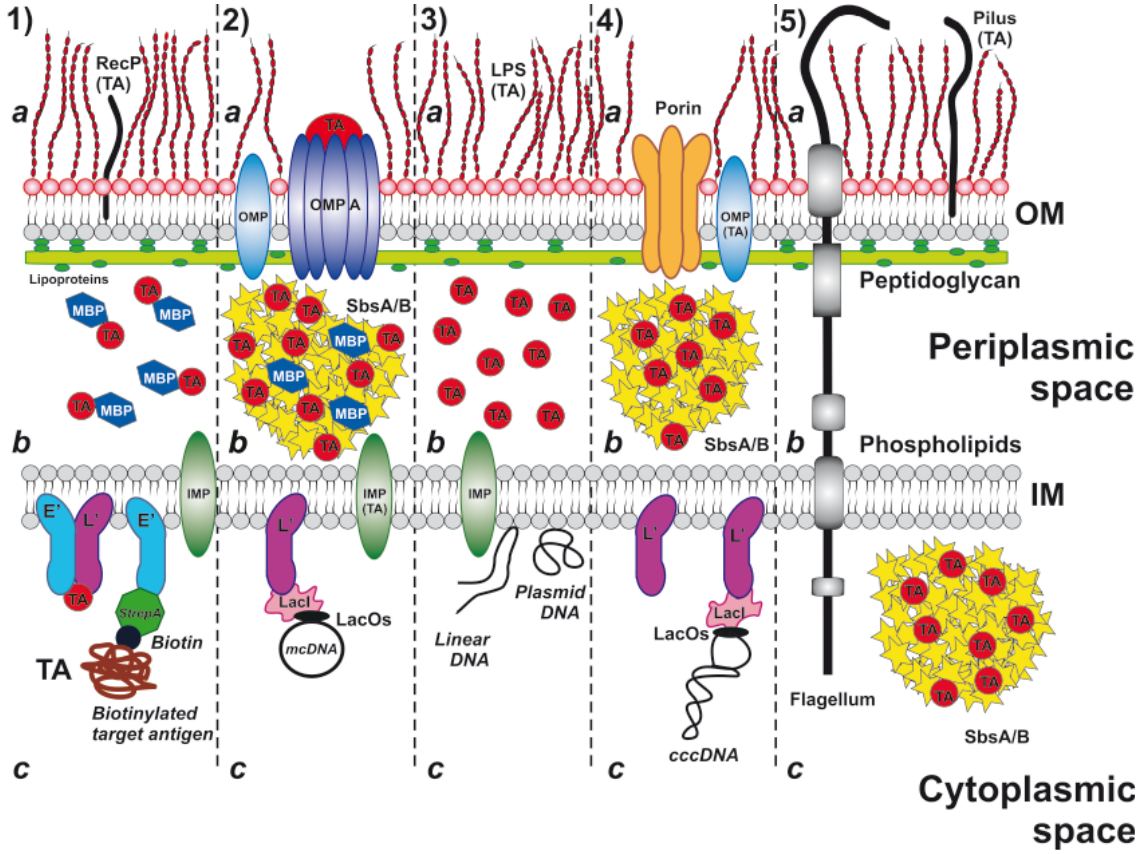


**Figure 1. Diversity of target Ag (TA) location in the different areas of the BG envelope.** 1) Recombinant or induced pilus (RecP) acting as TA (a); export of target Ag (TA) to sealed periplasmic space as maltose-binding protein (MBP) fusion (b); protein TA anchored into the inner membrane (IM) via E', L' or E' and L' anchor sequences or attachment of biotinylated TA to E'-membrane anchored Streptavidine (StrepA) (c). 2) Presentation of TA on the outer membrane (OM) surface as fusion protein with outer membrane protein A (OMP A) (a); export of TA to the sealed periplasmic space as MBP-sbsA or -sbsB S-layer fusion proteins (b); minicircle DNA (mcDNA) carrying the lac operator sequence (LacOs) anchored to the IM via L'-membrane anchored LacI (LacI-L') or inner membrane protein (IMP) acting as TA (c). 3) Lipopolysaccharide (LPS) acting as TA (a); export of TA to the sealed periplasmic space using a signal sequence that is cleaved off after transport, e.g. GIII, MBP signal sequence (b); loading of bacterial lumen with linear and cDNA plasmids making BG as carrier for DNA vaccines (c). 4) Outer membrane protein (OMP) acting as TA (a); export of TA to the sealed periplasmic space as sbsA or sbsB S-layer fusion proteins using a signal sequence that is cleaved off after transport, e.g. GIII, MBP signal sequence (b); DNA carrying the LacOs anchored to the IM via L'-membrane anchored LacI (LacI-L') repressor molecule (c). 5) Pilus acting as TA (a); periplasmic space (b); cytoplasmic space filled up with recombinant S-layer proteins carrying foreign TA (c).

## Plasmid DNA vaccines

Plasmids most commonly used for vaccination are double stranded DNA molecules encompassing two functional units, the eukaryotic expression cassette and the bacterial backbone (BB). The eukaryotic expression cassette also known as transcription unit comprises of promoters, enhancers and the polyadenylation signal necessary for successful expression of the desired gene(s) in the target cell. The BB of the plasmid in most cases

consists of the antibiotic resistance gene, an origin of replication (critical for plasmid DNA production inside the bacteria) and unmethylated phosphodiester-linked cytosine and guanine (CpG) motifs, which stimulate the innate immune response [95].

Genes encoding immunomodulatory proteins possessing the capacity to increase and modulate the immune responses driven against the specific Ag encoded on an expression plasmid can either be included on the same plasmid or administered separately via an additional plasmid(s). Genes encoding cytokines, chemokines, T cell co-stimulatory molecules and other molecules involved during the entire process of Ag-specific immune responses have been investigated for their capacity to stimulate and enhance the plasmid-encoded Ag-specific immune response [4,96-97].

Unmethylated CpG motifs are important components of DNA vaccines. They play an important role in the activation of the innate immune system of the host by binding to the TLR9 of professional APCs [98-99]. Because of their less frequent presence in vertebrates, unmethylated CpG motifs are considered to act as an alarm signal for the immune system during bacterial infection [95]. Moreover, recognition and binding of CpG motifs to TLR9 stimulate maturation, differentiation and proliferation of natural killer cells (NK), plasmacytoid DCs, macrophages and T cells, followed by secretion of cytokines polarizing the development of T cell immune responses toward Th1 type [98-99]. Furthermore, CpG motifs alone also act as Ag-independent endogenous adjuvant.

Antibiotic resistant genes represent an important part of plasmid DNA vaccines because of their significant role in selection of bacteria carrying relevant pDNA during the production process. The European Medicines Agency (EMA), formerly European Agency for Evaluation of Medicinal Products (EMEA), and Food and Drug Administration of the USA (FDA) strictly prohibit the use of penicillin and other β-lactam antibiotics during vaccine production processes due to serious biosafety risks posed by these sequences [100-102]. Traces of these antibiotics in administered vaccines might lead to serious allergic reactions and/or the resistance against these markers in environmental micro-organisms. Therefore the regulatory agencies, e.g. EMA and FDA recommend only the use of Kanamycin or Neomycin antibiotic resistant markers for the purpose of plasmid DNA vaccination or gene therapy as these aminoglycoside antibiotics are not widely used in medical practice [100-103]. Moreover, regulatory agencies strongly recommend full elimination of the use of antibiotic-resistance genes in plasmid DNA, as well as the use of antibiotics during DNA vaccine

production processes. Following these recommendations, our group focused on the development and the construction of novel plasmid DNA vaccines lacking plasmid BB e.g. minicircle DNA (mcDNA). Using of mcDNA together with BGs for future vaccine applications is discussed in the next section.

At present time only a few DNA vaccines have been approved in veterinary practice, e.g. vaccine against Infectious Hematopoietic Necrosis virus (IHNV) in Salmon inducing protective immunity, which leads to the improvements of animal welfare, and quality and quantity of food [104]; vaccine against West Nile virus in horses inducing efficient Ag-specific humoral and cellular immune responses capable to protect animals against infection [105-106]; xenogeneic DNA vaccine against dog melanoma, the first approved DNA vaccine against cancer, leading to development of Ag-specific humoral immune responses and survival prolongation [107]; and the most recently intramuscular DNA vaccination of pigs with plasmids encoding growth hormone releasing hormone, which stimulated Ag-specific humoral immune responses, enhanced the protection against *Mycoplasma hyopneumonia* and significantly decreased the mortality and the clinical disease associated with *Mycoplasma hyopneumonia* [108] . There is still a need for an intensive research for the development of DNA vaccines and their practical use in veterinary medicine. The requirement for the use of high doses of plasmid copies as a consequence of the low immunogenicity of current DNA vaccines represents one of the reasons responsible for the slow pace in the development and licensing approval of current DNA vaccines. This fact is most commonly attributed to the absence of efficient carrier systems capable to deliver specific DNA (Ag) to the target cells and stimulate them to induce effective Ag-specific immune responses [109]. Plasmid DNA vaccination strategy has a lot of potential due to its simplicity, versatility of application routes, modes of vaccine delivery, and the capacity to induce desired Ag-specific immune responses. However extensive investigations are needed for the development of novel delivery systems capable to target specific immune cells and increase the immunogenicity of delivered Ags [110].

**BGs-carrier of DNA encoded Ag**

BGs possess excellent loading capacity and can be filled with high amounts of nucleic acids [89]. Loading of BGs involves simple resuspension of powdered dried lyophilized BGs in a high concentrated plasmid DNA solution followed by extensive washing steps to remove

unbound plasmid DNA. The final amount of DNA present within the BGs depends on the concentration of the DNA solution used. Previous studies showed that using this method up to 6000 mid size plasmid copies can be stored within a single BG proving both the great loading capacity of BGs and the high efficiency of the used procedure for loading of BGs [12]. The interaction between plasmid or linear dsDNA and the cytoplasmic membrane of BGs is most likely due to an electrostatic interaction of negatively charged molecules of DNA and positively charged moieties (amine groups) present on the non polarized IM [12]. Moreover, plasmid DNA can be loaded and stored inside the BGs either by binding to biotinylated dextran or to polylysin present within the inner space of BGs [38]. Furthermore, BGs can be efficiently loaded by plasmid DNA using the simultaneous binding of a LacI repressor protein from the fusion complex of LacI and a hydrophobic IM-anchoring sequence (L' anchor) to a particular DNA sequence (a tandem repeat of a modified lactose operator sequence-the LacOs sites) of a specific self-immobilizing plasmid DNA (pSIP). Expression of the fusion complex LacI-L' membrane anchor leads to its incorporation to the IM of BGs. Plasmid DNA bound to the fusion complex through the LacOs sites is therefore immobilized within the bacteria and stays there during the E-lysis process yielding into BGs carrying pSIP in the interior [111-112]. To sum up, two potential mechanisms can be employed for using BGs to deliver DNA into the target cells. In a two step procedure BGs are first produced and lyophilized to obtain a dry-powdered form, and then BGs are loaded with high amounts of desired plasmid DNA [12,43,76-77]. Alternatively, a simple one step process for generation of BGs carrying specific plasmid DNA using the pSIP system has been developed. This process allows us to get DNA loaded BGs already before the lyophilization without the need of any additional step.

The pSIP technology has been recently further improved especially for DNA vaccines. One of the most critical points in DNA vaccine advancement is to develop a system capable to deliver only the target gene of interest without the transfer of other genetic information, e.g. antibiotic resistance markers and origin of replication. The BB sequences including the antibiotic resistance genes are considered as high biological safety risk for vaccination using plasmid DNA, therefore in the future vaccine development all these issues need to be addressed as a matter of priority. A novel advanced version of the pSIP technology based on mcDNA devoid of entities posing a biological risk to vaccinated subject was developed to overcome this hurdle in using BGs loaded plasmid DNA in vaccination. This improved version of pSIP is based on the ParA resolvase system capable to produce both mcDNA

bound to the IM receptor and the sister-pair miniplasmid DNA (mpDNA) which is released to the cytoplasm and expelled together with other cytoplasmic content to the culture media during the E-mediated lysis (FIGURE 2) [112].

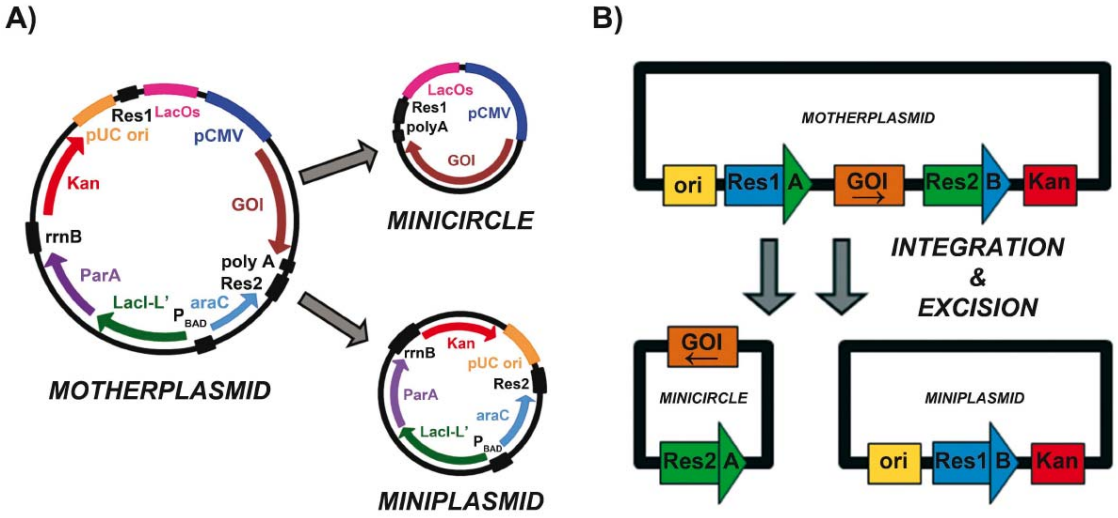


**Figure 2.** Advanced version of pSIP produces minicircle DNA without the antibiotic resistance genes anchored to the IM of BG envelopes via the LacOs - LacI-L' interaction. Mother plasmid after recombination is divided into miniplasmid comprising antibiotic resistant gene, one recombination site along with bacterial backbone, and into minicircle containing only LacOs necessary for binding to IM anchored LacI-L' and the gene of interest along with promotor, repressor system, polyA signal and one recombination site (A). Detailed schema of the parA mediated site specific recombination during production of minicircle DNA (B). araC, repressor/inducer of $P_{BAD}$ promoter; GOI, gene of interest; Kan, Kanamycin resistance gene; LacI-L', fusion protein of LacI repressor with truncated lysis protein of bacteriophage MS2 serving as membrane anchor L'; LacOs, modified lac operator sequence with high affinity to bind LacI; ParA, ParA resolvase gene; $P_{BAD}$, arabinose inducible promoter; pCMV, cytomegalovirus early promoter; polyA, SV40 late poly adenylation signal; pUC ori, origin of replication derived from pUC19; Res1, resolution site 1; Res2, resolution site 2; rrnB, transcriptional terminator sequence of 5s ribosomal gene.

Moreover, a modified system of mcDNA production using the endonuclease activity of *I-SceI* gene encoded from the parent plasmid for digestion of the mpDNA has been reported lately [113]. Additionally to our previous studies with BGs and the pSIP technology, a novel system involving ParA recombination, E-lysis and SNUC activity combined all at once is under investigation. The advantage of this new technique is the potential to get rid of unwanted mpDNA by the action of *SNUC* and to prove that BGs harboring mcDNA can be produced in one step process simplified even further (Muhammad and Kudela, personal communication). Combination of endonuclease activity along with the pSIP technology would further allow increasing the purity of BGs comprising specific mcDNA by digestion of all unwanted plasmids which could be present within BGs, e.g. non-recombinant mother plasmids and the ParA produced mpDNA. Moreover, implementation of this technique into

the practice would simplify the whole production process and eliminate additional necessary purification steps.

**BGs stimulate effective humoral and cellular immune responses**

The capacity of BGs to elicit specific humoral and cellular immune responses either against the bacterial strain used for BGs generation or the target Ag along with characterization of stimulated immune responses were investigated using a wide range of different types of experimental animals, e.g. mice, rabbits, pigs, possum, fish and cattle [43,46,49,51-52,54,114-115]. Different models of immunization schemes concerning BGs administration including intraperitoneal, intradermal, intramuscular, subcutaneous, intragastric, and aerogenic routes or intravenous immunization by DCs *ex vivo* transfected with DNA loaded BGs were performed to assess the induced specific humoral and cellular T cell immune responses [43,46-47,50-51,53,55,82,116].

Aerosol immunization of pigs with BGs derived from the lung pathogen *A. pleuropneumoniae* led to a significant increase of specific IgG titers together with enhanced ratio levels of CD4+/CD8+ lymphocytes [51]. Mutual co-incubation of APCs and *A. pleuropneumoniae* BGs with T cells isolated from blood of animals immunized with *A. pleuropneumoniae* BGs resulted in significantly higher proliferation of specific T cells compared to proliferation of T cells stimulated with APCs but without BGs. Furthermore, flow cytometry analysis of the co-stimulatory and antigen-presenting molecule expressions on the surface of porcine DCs after incubation with *A. pleuropneumoniae* BGs revealed their significant up-regulation. Observed fluorescence intensities of MHC class II molecules expressed on the surface of DCs incubated with BGs was at least one magnitude higher compared to non-stimulated DCs [82]. Moreover, entire analysis showed that the vaccination of pigs against respiratory diseases caused by *A. pleuropneumoniae* using an aerosol form of *A. pleuropneumoniae* BGs fully protects animals against clinical disease or lung lesions [51]. The capacity of BGs to activate the immune system of the host and protect the host from a lethal challenge with the bacterial pathogen was also detected after intragastric immunization of BALB/c mice with BGs prepared from EHEC. BG's induced humoral immune response and stimulated Ag-specific IFN-γ producing T cells were able to protect and enhance survival of animals immunized with a single-dose of BGs up to 86% after the challenge with a

heterologous EHEC strain. Furthermore, additional booster with BGs increased the level of survival up to 93% [53].

Intramuscular immunization of C57BL/6 mice with BGs prepared from recombinant *V. cholerae* carrying the major outer membrane protein of *Chlamydia trachomatis* induced local mucosal and systemic Th1 type specific immune responses. The enhanced Ag-specific T cell immune responses were confirmed by detection of elevated IFN-γ production by T cells isolated and purified from the mucosal genital area and spleen after stimulation in the presence of APCs and the specific Ag. Detailed analysis showed no significant difference in the IL-4 production by T cells from immunized animals after re-stimulation with the specific Ag. Moreover, Ag-specificity and functional efficiency of stimulated T cells was confirmed by the transfer of purified T cells from immunized animals to naïve animals followed by the challenge with specific bacterial pathogens. Obtained results showed that mice with adoptively transferred effector T cells were highly resistant and protected against the pathogen infection compared to the infected non-immunized naïve mice [44,47]. Moreover, capacity of BGs prepared from *Vibrio cholerae* expressing Porin B and polymorphic membrane protein-D proteins of *C. trachomatis* to induce long lasting immune responses was examined after intramuscular immunization of C57BL/6 mice. Strong anamnestic systemic and mucosal immune responses were observed in vaccinated animals. Immunized mice efficiently cleared pathogens within 12 days after intravaginal challenge with chlamydia inclusion forming units when compared to the control group. Furthermore, re-challenging of mice 98 days after resolution of the primary infection resulted in the recall of Ag-specific humoral and cellular immune responses indicating effective long-lasting protective immunity induced by BGs carrying multisubunit Ags [117]. Altogether, these results emphasize the capacity of BGs used as protein Ag carrier to significantly participate in the induction of a Th1 type specific T cell immune response after intramuscular immunization.

Therapeutic immunization of C57BL/6 mice with BGs prepared from *H. pylori* carrying recombinant Omp18 protein 4 weeks after three oral inoculations with a cocktail of 5 Iranian type-I Hp strains led to the lowest rate of infection compared to animals immunized with pure Cholera Toxin adjuvant and PBS. Moreover, statistically significant increase of Omp18-specific antibody titers and reduction in bacterial gastric colonization of the infected mice were observed after immunization with *H. pylori*-rOmp18 BGs compared to control mice [118].

Intramuscular immunization of BALB/c mice with *V. cholerae* and *E. coli* NM522 BGs loaded with plasmid DNA encoding gp63 of *Leishmania major* induced significantly increased Ag-specific humoral and cellular immune responses. Moreover, T cells isolated from the spleen of immunized animals restimulated with recombinant gp63 produced increased levels of IFN-γ but not of IL-4 indicating induction of a Th1 type Ag-specific immune response (Tabrizi and Lubitz, manuscript in preparation).

Intradermal and intramuscular immunization of BALB/c mice with *M. haemolytica* BGs loaded with pCMV encoding β-galactosidase induced efficient humoral and cellular responses against the delivered Ag. The humoral response against the Ag delivered as plasmid DNA within BGs was at least one order of magnitude higher than the response observed after immunization with naked DNA. Evaluation of stimulated Ag-specific T cell responses displayed the presence of both Th1 and Th2 components of the induced immune response, but further analysis showed a shift to the predominant Th2 response suggesting a modulation of the Ag specific Th response by the bacterial strain used for preparation of BGs [43].

Furthermore, studies with mouse professional APCs, e.g. macrophages and DCs confirmed an excellent capacity of BGs to deliver plasmid DNA encoding a specific Ag to the target cells resulting in the expression of Ags within the targets with transfection efficiency up to 85% [12,43]. Besides, intravenous immunization of mice with bone-marrow derived DCs *ex vivo* transfected with *M. haemolytica* BGs loaded with pCMV-β-galactosidase induced specific anti-β-galactosidase Ab responses in all vaccinated animals. Moreover, an enhanced number of IFN-γ producing T cells recognizing the peptide comprising the most immunogenic MHC class I epitope from β-galactosidase was observed after vaccination [43]. Similarly to the results obtained after treatment of porcine DCs with BGs from A. *pleuropneumoniae*, co-incubation of mouse DCs with *M. haemolytica* and *V. cholerae* BGs stimulated an increased expression of antigen-presenting and co-stimulatory molecules on the surface of DCs [43,47,82]. Quantitative analysis of BG's loading capacity showed that the relatively low DNA concentration (50 plasmids per BG) is sufficient for efficient DNA delivery to the target cells leading to transfection efficiency up to 82% [77]. Taking into account the excellent DNA loading capacity of BGs up to 6000 plasmid copies per single BG and the lowest DNA concentration sufficient enough to successfully transfect target cells (50 per BG), the high loading capability of BG's inner space suggests the use of BGs as delivery vehicle for multiple types of plasmids encoding various types of Ags and/or other types of active immunomodulary substances.

Evaluation of immune responses induced by BGs prepared from different bacterial strains using various animal models and different target cells indicates unique properties of BGs to stimulate effective humoral and cellular immune responses against the bacterial strain used for BG preparation and Ag carried and delivered by BGs to the target cells sufficient enough to protect tested animals after the challenge infection with the specific strain or Ag, and/or to induce effective immune responses against Ags delivered by DNA loaded BGs (Table 2). Application of BGs to the target animals using different preparation forms, e.g. aerosol, food, liquids, and diverse routes for immunization together with the facts concerning BG's immunostimulatory capabilities mentioned above point directly to the prospective use of BGs in the development of polyvalent vaccines. Assessment of results from animal experiments obtained after immunization with BGs will help us to select the most suitable bacterial strain for preparation of BGs in order to stimulate the most effective type of cellular and humoral immune responses against the specific type of disease (pathogen). Summarized overviews of immune responses obtained from studies using BGs as vaccine candidate and antigen carrier are shown in Table 3 (human applications), Table 4 (veterinary applications), and Table 5 (tissue culture studies).

**Table 2. Overview of immune responses stimulated by BGs and Ag delivered by BGs.**

| Immune response | Model | Ref. |
|---|---|---|
| ***Innate*** | | |
| Induced expression of antimicrobial peptides | Keratinocytes: psoriasin, β-defensin | [140] |
| Increased secretion of pro-inflammatory cytokines | PBMCs, DCs: IL-6, IL-8, IL-12, TNF-α, IFN-γ | [Lubitz and Haslberger, personal communication] |
| ***Adaptive / Humoral*** | | |
| Antigen-specific immune response | *E. coli* HIV-1 RT; *E. coli* HIV-1 gp41; *E. coli* ZP2; *V. cholerae* pDNA-GP63 (Th1); *M. haemolytica* pCMVβ (Th0/Th2); *V. cholerae* MOMP (Th1); *V. cholerae* M1-2 (Th1); *E. coli* NTHi OMP26 (Th1) | [33,43,54,93,141] [Tabrizi and Lubitz, manuscript in preparation] |
| Protective immunity against homologous challenge | *V. cholerae* M1-2; *EHEC*; *A. pleuropneumoniae* | [51,53,141] |
| Long-lasting protective immunity | *V. cholerae* | [117] |
| Cross-protective immunity against heterologous serovars | *V. cholerae* CTA2B-MOMP; *K. pneumoniae*; *E. coli* NTHi OMP26; *P. multocida*; *V. cholerae* TCP | [33,46-47,52,93,121] |
| | | |
| ***Adaptive / Cellular*** | | |
| $CD4^+$ | *A.pleuropneumoniae*; *V. cholerae* rGP63; *E. coli* ZP2; *M. haemolytica* pDNA-CMVβ | [43,51,54][Tabrizi and Lubitz, manuscript in preparation] |
| $CD8^+$ | *EHEC* | [53] |
| $CD4^+/CD8^+$ | *E. coli* HIV-1 gp41; *V. cholerae* CTA2B-MOMP | [33,47] |

PBMCs, peripheral blood mononuclear cells; DCs, dendritic cells; MOMP, major outer membrane protein; OMP, outer membrane protein

**Table 3. Overview of immune responses against BGs used as empty cell envelope, subunit or DNA vaccine for human medicine.**

| Target Ags/ Location in BGs | Disease caused | BG carrier | Animal Model | Route of immunization | Immune response | Ref. |
|---|---|---|---|---|---|---|
| Cell envelope TCP | Cholera | *V. cholerae* | Mouse | Intraduodenal | Vibrio-specific humoral response. | [55] |
| Cell envelope TCP | Cholera | *V. cholerae* | Rabbits | Subcutaneous/ Intramuscular | Protective humoral response; cross-serogroup protective immunity. | [45] |
| Cell envelope TCP | Cholera | *V. cholerae* | Rabbits | Oral/RITARD model | Cross-protective humoral response; dose-dependent protective immunity against intraduodenal challenge. | [46] |
| Cell envelope | Gastritis, Dispepsia, Ulcers, Cancer | *H. pylori* | Mouse | Oral | Significant reduction of bacterial load; 15/20 animals protected without use of adjuvant; complete protection after coadministration with mucosal adjuvant. | [116] |
| Cell envelope | Pneumonia | *K. pneumoniae* | Piglets | Subcutaneous | Ab titers comparable to titers after infection with virulent bacteria; cross-reactivity to related subspecies. | [33] |
| Cell envelope | Typhoid-like disease | *S. typhimurium* | Mouse | Oral | Significantly increased survival of vaccinated animals. | [33] |
| Cell envelope | Bloody diarrhea | *E.coli* | Mouse | Intraperitoneal/ Subcutaneous | Ag-specific humoral response; increased survival time. | [33] |
| Cell envelope | Bloody diarrhea | *E.coli* | Rabbits | Subcutaneous | Ag-specific humoral response; elicited T-cell stimulation. | [33] |
| HIV-1 gp41 IM anchored | AIDS | *E.coli* | Mouse | Intraperitoneal | Cellular immune response. | [33] |
| HIV-1 RT- IM anchored | AIDS | *E.coli* | Mouse | Intraperitoneal/ Subcutaneous | Ag-specific humoral response. | [33] |
| rGP63 of Leishmania- IM anchored | Leishmaniasis | *V. cholerae* | Mouse | Intramuscular | Significant humoral and cellular (Th1 type) immune response. | [Tabrizi and Lubitz, manuscript in preparation] |
| *C. trachomatis* rMOMP/OMP2 -IM anchored | Chlamydiosis, Genital infections | *V. cholerae* | Mouse | Intramuscular | Local mucosal and systemic Th1 type specific immune response. | [141] |
| CTA2B- IM attached | Chlamydiosis, Genital infections | *V. cholerae* | Mouse | Intramuscular/ Intravaginal | Mucosal and systemic humoral, and Th1-type cellular immune response; cross-protection against heterologous Chlamydia serovars. | [47] |
| sbsA-Omp26 *H. influenzae* | Influenza | *E.coli* | Mouse | Intraperitoneal | Omp26-specific humoral response; significant titter of Omp26-specific IgG. | [90] |
| OmpA-HbcAg-149- IM,OM anchored | Hepatitis B | *E.coli* | Mouse | Subcutaneous | Significant HbcAg-149 specific humoral response. | [50] |
| pCMV-β-galactosidase | | *M. haemolytica* | Mouse | Intradermal/ Intramuscular | Elicited β-galactosidase humoral and cellular specific immune responses. | [43] |
| Sbs A-Omp26 NTHi cytoplasm | Respiratory tract infections and otitis media | *E.coli* | Rats | Intestinal / Intraduodenal | Omp26-specific humoral immune response. | [93] |
| MBP-Omp26 NTHi periplasmic space | Respiratory tract infections and otitis media | *E.coli* | Rats | Intestinal / Intraduodenal | Omp26-specific humoral immune response. | [93] |

IM, Inner membrane; OM, outer membrane; TCP, Toxin-coregulated Pilus

**Table 4. Overview of immune responses against BGs used as empty cell envelope, subunit or DNA vaccine for veterinary medicine.**

| Target Ags/ Location in BGs | Disease caused | BG carrier | Animal Model | Route of immunization | Immune response | Ref. |
|---|---|---|---|---|---|---|
| Cell envelope | Porcine pleuropneumonia | *A. pleuropneumoniae* | Pig | Intramuscular/ Homologous aerosol challenge | Significant Ag-specific humoral immune response; full protection against clinical disease and lung lesions; prevented colonization of the respiratory tract. | [48] |
| Cell envelope | Porcine pleuropneumonia | *A. pleuropneumoniae* | Pig | Aerogenic/ Homologous aerosol challenge | Significant Ag-specific humoral immune response; increased $CD4^+/CD8^+$ ratio; complete protection against challenge infection. | [51] |
| Cell envelope | Bovine respiratory disease | *M. haemolytica* | Cattle | Subcutaneous/ Intratracheal challenge | Protective immunity against homologous challenge; cross-reactivity to various *Pasteurella* serotypes. | [52] |
| Cell envelope | Bovine respiratory disease | *P. multocida, M .haemolytica* | Rabbit | Subcutaneous | Significant Ag-specific humoral immune response; cross-reactivity to various *Pasteurella* serotypes. | [121] |
| Cell envelope | Bovine respiratory disease | *P. multocida, M .haemolytica* | Mouse | Intraperitoneal/ Homologous challenge | Significant Ag-specific humoral immune response; cross-reactivity to various *Pasteurella* serotypes; protective immunity against homologous challenge. | [121] |
| Cell envelope | Gastroenteritis, hemorrhagic colitis, hemolytic uremic syndrome | EHEC | Mouse | Oral, Intragastric/ Heterologous challenge | Significant Ag-specific cellular and humoral immune response; high rates of protection after challenge (86,6%-93,3%) in comparison to negative control (26,7%-30%). | [53] |
| ZP2/Periplasmic space | | *E.coli* | Possum | Nasal/ Conjunctival mucosa | Ag-specific cellular and humoral immune response; reduced possum fertility. | [54] |
| Cell envelope | Edwardsiellosis | *E. tarda* | Fish | Oral | Significant systemic and mucosal Ag-specific humoral immune response. | [126] |
| Cell envelope | Edwardsiellosis | *E. tarda* | Mouse | Oral/ Intragastric challenge | Ag-specific humoral immune response; significant increases of $CD3^+$, $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ T cell populations; significantly elicited rates of protection after challenge (86,7%) compared to negative control (33%). | [142] |
| Cell envelope | Hemorrhagic septicemia | *A. hydrophila* | Fish | Oral/ Challenge | Ag-specific humoral immune response; increased rates of protection after challenge compared to negative control. | [143] |
| GADPH from *A. hydrophila* | Hemorrhagic septicemia | *E.coli* | Fish | Intraperitoneal/ *in vivo* release of Ag/ Challenge | Ag-specific immune response; high rates of protection after challenge (>80%). | [115] |
| Cell envelope | Gastroenteritis, hemorrhagic colitis, hemolytic uremic syndrome | EHEC | Mouse | Rectal/ Heterologous challenge | Significant Ag-specific cellular and humoral immune response after a single rectal immunization; full protection of mice after heterologous LD50 challenge. | [Mayr and Lubitz, submitted manuscript] |

Ag, antigen; EHEC, enterohemorrhagic *E.coli*; ZP, zona pellucida

**Table 5. Overview of *in vitro* studies with BGs used as empty cell envelope, subunit or DNA vaccine delivery system.**

| Target Ags/ Location in BG | BG carrier | Model | Proof of principle | Outcome | Ref. |
|---|---|---|---|---|---|
| pEGF/ Cell envelope | *E. coli* *M. haemolytica* | Human DCs | Tissue culture | Efficient expression of delivered heterologous gene (>77%); no toxic impact on DCs | [76] |
| pEGF/ Cell envelope | *M. haemolytica* | Mouse DCs<br>Mouse macrophages | Tissue culture | Efficient expression of delivered heterologous gene; enhanced expression of MHC molecules and co-stimulatory molecules by DCs | [43] |
| pEGF/ Cell envelope | *E. coli* | Mouse macrophages | Tissue culture | Efficient expression of delivered heterologous gene by target cells; determination of BG's high loading capacity (up to 6000 plasmid copies per single BG) | [12] |
| pEGF/ Cell envelope | *M. haemolytica* | Melanoma cells | Tissue culture | High expression levels of delivered gene after incubation with low amount of BGs (1 BG per single cell) | [77] |
| Cell envelope | *A. pleuropneumoniae* | Porcine DCs | Tissue culture | Significant enhancement of antigen-presenting and co-stimulatory molecules on DCs; significant increase of BG's specific T cell response | [82] |

DCs, dendritic cells

## BGs-veterinary vaccines

The route of immunization is of great importance in veterinary practice. The most preferred way of vaccine application is the mucosal administration like oral, intranasal, intraocular or aerogenic immunization. Previously performed studies using BGs as vaccine showed that the oral vaccine administration needs almost ten times higher doses of BGs compared to the dose applied intramuscularly, while intranasal and intraocular applications requires only the double doses than what is sufficient for intramuscular injection [119]. The mucosal route of immunization has several advantages over other routes because of easy administration, reduced side effects and the high potential for frequent boosting [120]. Immunizations with BGs produced from various pathogenic Gram-negative bacterial strains have been studied using different animal models [14,91]. BGs prepared from *A. pleuropneumoniae* were used to protect pigs against actinobacillosis. Animals vaccinated intramuscularly with *A. pleuropneumoniae* BGs were not only protected against the clinical disease but were also protected against colonization of lungs by *A. pleuropneumoniae* upon exposure [49]. Moreover, pigs immunized with *A. pleuropneumoniae* BGs aerosols developed sterile immunity against the lung pathogen [51]. The capacities of *P. multocida* and *M. heamolytica* BGs to stimulate specific immune responses against the examined pathogen were

tested in rabbits and mice. Administration of *P. multocida* and *M. heamolytica* BGs using distinct immunization routes-subcutaneously (rabbits) or intraperitoneally (mice) led to development of specific antibodies against the strains used for generation of BGs. Further, study results showed that the induced antibodies exhibited cross-reactivity against other *Pasteuella* serotypes and field isolates. Moreover, intraperitoneal immunization of mice with BGs fully protected vaccinated animals against challenge with a virulent *P. multocida* depending on the immunization dose. Obtained results demonstrate dose dependent capacity of *Pasteurella* BGs to induce protective immunity [121]. Furthermore, cattle immunized with *M. heamolytica* BGs developed better protective immunity compared to commercially available vaccines [52]. BGs from *E. coli* O78:K80 and other serotypes provided protection to one day old chicks against colibacillosis when administered intramuscularly while the chicks vaccinated intranasally showed similar mortality as observed in the controlled group when challenged with the specific pathogen [14]. An orally administered autogenous *E. coli* vaccine made of BGs prepared from three *E.coli* isolates *E. coli* 078K80, *E. coli* 7155/06 and *E. coli* 115/7 was compared with an intramuscularly administered conventional vaccine. Observed results clearly demonstrated that the BGs vaccine was at least as well tolerated as the conventional vaccine under standard commercial production conditions without showing any adverse effect on animal health. Low numbers of losses (< 2.3%) were observed in both groups of vaccinated animals. Moreover, no acute infection was detected after administration of the BGs vaccine compared to the conventional vaccine. (Lubitz, unpublished data). Further, the immunization of piglets with *K. pneumoniae* Kpn-3 BGs elicited antibody titers comparable to that seen after an infection by virulent bacteria. The sera from *K. pneumoniae* Kpn-3 BGs infected piglets also showed reactivity against the human *K. pneumoniae* strain A565 indicating cross-protective immunity with other serotypes [33]. *V. cholerae* BGs prepared from O1 or O139 strains were evaluated as vaccines in the reversible intestinal tie adult rabbit diarrhea (RITARD) model. Rabbits orally immunized with different doses of *V. cholerae* BG formulations were fully protected against the disease [46].

**BG-fish vaccine**

The growing importance of aquaculture, more particularly fish farming, has an industrial boom due to an increased demand of marine food on the nutrition market. The majority of industrial farm animals including fishes suffer from physical stress because of

their growth in unnatural environment which is usually overcrowded. These stressful conditions make them more prone to bacterial infections like Edwardsiellosis and Vibriosis, which in most cases result in massive economic losses. Continuous treatment of these diseases with antibiotics leads to the development of antibiotic resistant bacterial strains. Recently there is a growing concern among end consumers regarding drug residues in meat of industrial farm animals including fishes. These facts also point out why the prophylaxis of infectious diseases in farmed marine and freshwater fish is so critical, as well as the importance of fish vaccine development [122].

*Edwardsiella tarda* is a Gram-negative bacterium causing Edwardsiellosis in both fresh and marine fish, e.g. Catfish, Tilapia, Eel, and Chinook salmon. This disease is characterized by the septicemia with extensive skin lesions and leads to massive economic losses [123]. So far, there is no effective vaccine available against this disease due to wide range of *E. tarda* serotypes [124]. BGs prepared from *E. tarda* represent a novel potential system for design and development of a prospective vaccine applicable in farmed fish industry. Intraperitoneal immunization of Tilapia fish (*Oreochromis mosambicus)* with *E. tarda* BGs provided a higher degree of protection against Edwardsiellosis, compared to the group injected with formalin killed *E. tarda* [72]. Furthermore, *E. tarda* BGs administered orally to Olive Flounder (*Paralichthys olivaceus*) proved to be an ideal vaccine candidate eliciting both systemic and mucosal immune responses. Moreover, both studies confirmed that immunization with BGs is simple and less stressful to vaccinated fish of any size [122,125-127]. Recently BGs from another important fish pathogen *Vibrio anguillarum* were produced for future animal studies to counter the most serious fish disease named Vibriosis [128]. Although the use of BGs as a carrier of DNA vaccines regarding the application in fish was not intensively studied in last decades, results obtained from previously performed animal immunization studies using BGs together with the recent outcomes from investigations concerning fish pathogens indicate prospective use of BGs also in the development of fish vaccine. Furthermore, the BG system has been used to design a novel type of attenuated fish vaccines combining live attenuated *V. anguillarum* successfully used to induce cross-protective immunity against *Vibrio* pathogens [129] and BG technology. In this novel approach the attenuated bacteria carrying an *in vivo* inducible lysis gene *E* will be administered orally to the target fish population followed by specifically induced expression of gene *E* within the fishes, which will lead to the production of BGs from attenuated bacteria *in vivo*. This new vaccine system provides two major benefits. First, the BG technology

applied to the selected attenuated pathogen will guarantee no reversal to the native pathogenic form, and second the target expression of foreign recombinant Ags in the cytoplasm or their incorporation into the membrane of the host pathogen selected for immunization might serve as multivalent vaccine and stimulate immune responses against both delivered Ags and the pathogen. This type of multivalent vaccine was recently tested *in vivo* using a non-pathogenic recombinant *E. coli* as live vector capable to release expressed recombinant heterologous proteins *in vivo* after E-mediated lysis of the carrier vector. A heterologous Ag, the *gapA* gene encoding the protective GAPDH from the fish pathogen *Aeromonas hydrophila* LSA34, was produced in the live vector under control of the strong promoter $P_{T7}$, while the lysis gene *E* was tightly regulated by the iron-responsive promoter $P_{viuB}$. Intraperitoneal vaccination of Turbot (*Scophatalmus maximus*) followed by the induction of E-mediated lysis successfully led to the release of target Ag *in vivo* and the stimulation of protective immune responses. More than 80% of fishes immunized with the recombinant live vector expressing the Ag survived the challenge with pathogenic *Aeromonas hydrophila* LSA34, 30 days post vaccination [115]. These results indicate the future exploitable potential of the novel vaccination system combining the features of the BG technology and live bacterial vectors.

**BG-immunocontraceptive vaccine**

Immunocontraception was developed in the past decades to control the fertility of target subject through the immune system. Identification of a specific target Ag involved during reproduction of chosen subjects plays a critical role to reach the final contraceptive effect caused by induced Ag-specific immune responses. Unfortunately, the majority of newly identified Ags applicable as an immunocontraceptive vaccine has shown poor immunogenicity. To improve their potential to stimulate efficient humoral and cellular immune responses several modifications of the native molecule by coupling Ags with adjuvants and antibodies, or binding Ags to immunogenic carriers were performed and investigated [130-131]. Moreover, poor immunogenicity of selected Ags might be improved by using a system which combines the features of an adjuvant and an efficient delivery vehicle. Hence BGs carrying recombinant immunocontraceptive Ags were used to control the population of brush tail possums (*Trichosurus vulpecula*) in New Zealand. Brush tail possum is one of the most serious pest animals in New Zealand causing enormous economic and environmental damage and is responsible for the spread of Bovine Tuberculosis in wild life

population. The challenge for the development of a successful immunocontraceptive vaccine against this important pest not only lies in the identification of reproductive responsible target Ags as mentioned above, but also on the efficient delivery of the vaccine to the natural environment of the pest. Zona pellucida (ZP) protein 2 and 3 represent major targets of choice for controlling the possum population using the immunocontraception approach, however their delivery to the pest in wild life is still questionable. Recently the ZP2-N-terminal and ZP2-C-terminal proteins were fused to the maltose-binding protein followed by the expression and transportation into the periplasmic space of the *E. coli* NM522. Generated BGs were used to deliver the Ags into natural environment of the wild life of the target animal subjects. Female possums were immunized with prepared vaccine constructs mucosally through both nasal and conjunctival routes, and were examined after superovulation and artificial insemination. The possums immunized with ZP2-C-terminal protein constructs showed a significant drop in fertilization of eggs compared to the control group immunized with plain BGs [54], while the ZP2-N- terminal protein constructs showed no changes in fertilization levels. Moreover, it was observed that the BGs carrying the ZP3 Ag can induce strong humoral and cellular immune responses in the target species making them ideal for aerosol administration and/or delivery in the form of bait [94].

**Expert commentary**

Improvement of current vaccines or development of novel types of vaccines against the global diseases including infectious diseases caused by pathogenic microorganisms still represents a major task necessary to be successfully solved in order to prevent an increased risk of widespread epidemics, accidental outbreaks but also to decrease the incidences of diseases which occurrence can be eliminated or cured with known medicines. Novel advanced systems encompassing in future developed drugs and vaccines should comprise minimal undesired side effects but highly efficiencies, and have to be available for all people especially for those living in challenged conditions, and in less developed countries and poverty.

Majority of bacteria express potent toxins causing systemic damage of a wide range of tissues leading without particular treatment to diseases with severe outcomes and might produce causalities in both humans and animals especially in countries with less prosperous economies. Number of treatment strategies how to eliminate and minimize consequences

caused by bacterial infections were studied intensively over the past decades. Unfortunately, the antibiotic treatment is not effective or potent enough for the most important pathogens. Moreover, increased consumption of antibiotics by consumers even without the relevant need is getting popular for treatment of banal diseases and leads to increased rates of microbial drug resistances. Therefore European and American medical regulatory agencies due to this serious biosafety risk strictly recommend minimizing prescriptions of antibiotics without the significant need, and supporting progress and search for new types of disease treatment and vaccines development against severe bacterial infections allowing to decrease the amount of antibiotics used. However the final licensing of new products and their approval for clinical testing are getting strictly regulated with focus on their definition and safety.

BGs represent an efficient carrier system for delivery of protein, subunit or DNA encoded Ags endowed with intrinsic adjuvant properties capable to stimulate the innate and the adaptive immune system. BGs have a unique ability to induce specific cellular and humoral immune responses either against the bacteria used for their generation or the target Ags loaded inside the BG-envelopes. Moreover, the presence of unaltered BG surface structures efficiently enhances the immune response against poorly immunogenic Ags without the need of an extra adjuvant. A great advantage of BGs used as carrier of subunit vaccines is their simple production and loading with target Ags, making the production process more efficient and at lower costs. BGs can be produced very easily by fermentation in disposable or conventional steel fermenters of various sizes in research laboratories or in large GMP units of pharmaceutical industry. Meeting the modern requirements of rapid vaccine production the initial engineered working stocks of BGs vaccine candidates can easily produce large quantities of a BGs vaccine in a short period of time at low costs which makes the BG system ideal for use in veterinary and human vaccines. Furthermore, BG's safe vaccine profile poses no risk of reversal to the pathogenic form and minimizes the risk of horizontal gene transfer.

The route of administration is of great importance in both human and veterinary practice. Animal studies performed with BG vaccines using different models have shown the possibility of administration through various routes including the mucosal which is the most practical way for immunization of large populations as it is safe, less expensive and easily distributable worldwide. For example, a single oral [53] or rectal (Mayr and Lubitz, submitted manuscript) immunizations of mice with EHEC BGs prepared from a pathogen of food-borne diseases causing severe harms, especially in children and the elderly is equally protective as double immunizations after the lethal challenge in mice. This implies the possibility to

develop a novel efficacious single dose mucosal BGs vaccine using a simplified immunization regimen. These and other results indicate a high potential of BGs in mucosal administration without the need of an additional adjuvant making the immunization product more simple and affordable. Moreover, rectal vaccine administration in form of suppository is easily applicable also for children and might avoid the risk of target active substance/Ag degradation by stomach acid and proteolytic enzymes when administered orally. Besides mucosal administration of BGs, recent study showed that intramuscular immunization of mice with a BGs-based multisubunit chlamydial vaccine is capable to stimulate efficient local mucosal and long-term systemic cellular and humoral immune responses.

BGs can be generated from probiotic *E. coli* Nissle 1917, non-pathogenic and pathogenic Gram-negative bacterial strains, therefore selection of specific bacteria for generation of BGs prepared as bacterial vaccine only or as a carrier of single or multisubunit protein Ags or DNA should be based on the character of the target disease. For development of a vaccine against a single pathogen, the BGs made from that particular pathogen possessing the intact surface structural Ags in their original state along with their natural adjuvant properties related to the presence of various pathogen-associated molecular patterns such as LPS, monophosphoryl lipid A, flagellin and others on the BG envelope is functionally contributing to efficient induction of both innate and adaptive immune responses against the target pathogen. Furthermore, these natural adjuvant properties of BGs along with no cytotoxic and genotoxic impact of BGs on the viability and metabolic activity of cells recognizing BGs enhance the stimulated immune response against the Ag(s) after administration of a single or multisubunit BGs vaccine.

Altogether, data obtained from *in vivo* and *in* vitro studies performed over the past years indicate that BGs represent a promising, safe, cost effective and versatile multipurpose vaccine platform for application in a wide range of preventives and therapeutics in human and veterinary medicines.

## Five-year view

Additional confirmation and assessment of the immune responses stimulated by administration of BGs alone or as carrier of subunit protein Ags or DNA can be expected from planned animal and human clinical studies. Further investigations will be partially

aimed on the generation of BGs from important bacterial pathogens infecting both the farm animals and the pets, causing spread of foodborne and zoonotic diseases, respectively. Novel Ag delivery by BGs are going to be continuously evaluated *in vitro* as well as using appropriate animal model. We are aware that the presence of various intact PAMPs on the BG envelopes will require a complex regulatory approval process, but according to our safety data and previously obtained results we expect to start GMP production of an animal BGs vaccine and market it within the period of next five years. Concurrently our studies focus on the delivery of tumor Ags by BGs to DCs and tumor cells to determine whether this approach can be useful in tumor therapy as the PAMPs, e.g. peptidoglycan and LPS not only stimulate professional APCs but are also capable of providing stimulatory signals to non-professional APCs such as tumor cells. Adjuvant properties of BGs positively affect maturation and Ag presentation by DCs and lead to stimulation of effective Ag-specific T cells hence increase the recognition of tumor Ags presented on the surface of tumor cells by CTLs. The selection of the right envelope will be based on the capacity to stimulate proper cytokine secretion by DCs and also by the capability to stimulate Ag-specific T cells from naive cells. These concepts are expected to lead to phase I clinical trials and to an application of the system to the tumor treatment of patients with malignant melanoma side by side with the animal study oriented for the treatment of melanoma in pets.

Moreover, our recently obtained results demonstrated a high capacity of primary human conjunctiva-derived epithelial cells to internalize BGs with no cytotoxic effects on the cell viability and metabolic activity without restrictions to the bacterial species used for their preparation. The eye mucosa represents a prospective route for the vaccine administration having the capacity to induce both efficient systemic and mucosal Ag-specific immune responses. Eyedrop immunization with BGs carrying specific subunit protein Ags, DNA or drugs might be useful for future therapeutic ocular surface applications and an eye-specific disease vaccine development. Future investigations would help to progress the development of a microbial-mediated ocular disease vaccine and drug carriers.

**Box 1. Advantages of the Bacterial Ghost System**

- BGs can be produced from a wide range of non-pathogenic, pathogenic and probiotic Gram-negative bacteria.
- Large ability of the BG system for the selection of suitable bacterial candidates in vaccine development for veterinary and human applications.
- Safe, non-living system with absolutely no risk of reversal to the native pathogenic form.
- No potential hazard for horizontal gene transfer of plasmid encoded antibiotic resistance genes or pathogenic islands to the recipient.
- Capacity to serve as a natural adjuvant because of the intact morphological, structural and antigenic surface components of their living counterparts and to provide immunostimulatory signals to target cells.
- High potential to target various histological types of cells; recognition by a wide range of cells including dendritic cells, macrophages, tumor cells, endothelial cells and epithelial cells.
- No cytotoxic and genotoxic impacts of BGs on the viability and metabolic activity of cells recognizing BGs.
- Miscellaneous possibilities for an emplacement of target Ag within the BG envelope complex - inner membrane, periplasmic space, outer membrane, lysis hole.
- Ability to deliver chemotherapeutic drugs without modification of its pharmacological properties.
- Various possibilities of BGs administration-oral, aerosol, intradermal, intramuscular, intravenous, subcutaneous, intraperitoneal, intragastric, rectal, intravaginal.
- Immunizations with BGs induce strong humoral and cellular immune responses; cross-protective immunity against heterologous serovars and long-lasting protective immunity.
- High protection rates after mucosal or systemic administration without any addition of adjuvant.
- Simple and high dose manufacture process; production of BGs can be easily and quickly performed either in disposable fermenters, small lab steel fermenters or in large scale fermenters.
- Fermentation technology for BGs production allows the use of the system in a broad number of differently developed countries.

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# Appendix

## A.1 Removal of mutated mobilization sequence (MobM) of plasmid pGLysivb

### A.1.1 Introduction

It was an aim to get rid of all unnecessary sequences present on the lysis plasmids that are used in generation of BGs. Mobilization sequence (Mob) protein is a relaxase with autoregulation properties, the mob protein recognizes and binds to the 52bp region which contains the transfer origin (*oriT*) known as recombination site A (RSA) and promoters of *mob* gene [1]. These sequences are found in plasmid pGLysivb a derivative of pBBR1MCS5 [2] although the *mob* gene present on pGLysivb is mutated (MobM), but still it is desirable to remove the whole gene including the 52bp sequence which include the promoters of *mob* gene which is necessary for the mobilization of plasmid. Thus a strategy has been made to remove the MobM gene along with the gentamycin resistance cassette which is replaced by the amplified gentamycin resistance gene devoid of any extra sequences.

### A.1.2 Procedure

#### A.1.2.1 Cloning of plasmid pGELysR

In order to remove the MobM sequence from the plasmid pGLysivb (6201bp) (Fig.1), the plasmid pGLysivb was digested with *Apa*I and *Sna*I to remove 2238bp fragment containing the gentamycin resistance cassette and the MobM sequence. The 3683bp fragment of pGLysivb was gel purified using Purelink$^{TM}$ Quick Gel extraction kit (Invitrogen). In parallel the 1197bp fragment including the gentamycin resistance gene was amplified through PCR using primers GentFowr(*Apa*I) 5'- ATA *GGG CC'C* GGT ACC CAG CTT TT- 3' and GentRev(*Sna*I) 5'- ATA *GTA' TAC* TTA GGT GGC GGT ACT TGG GTC- 3' (Restriction sites Italicized) and plasmid pGLysivb as a template. The newly synthesized 1197bp PCR product containing gentamycin resistance cassette was digested with *Apa*I and *Sna*I (*Bst1107*I) both from (Fermentas) and subsequently cloned into the 3683bp fragment of

linearised pGLysivb to get plasmid pGELysR (5060bp) the cloning strategy is explained in detail in (Fig.1).

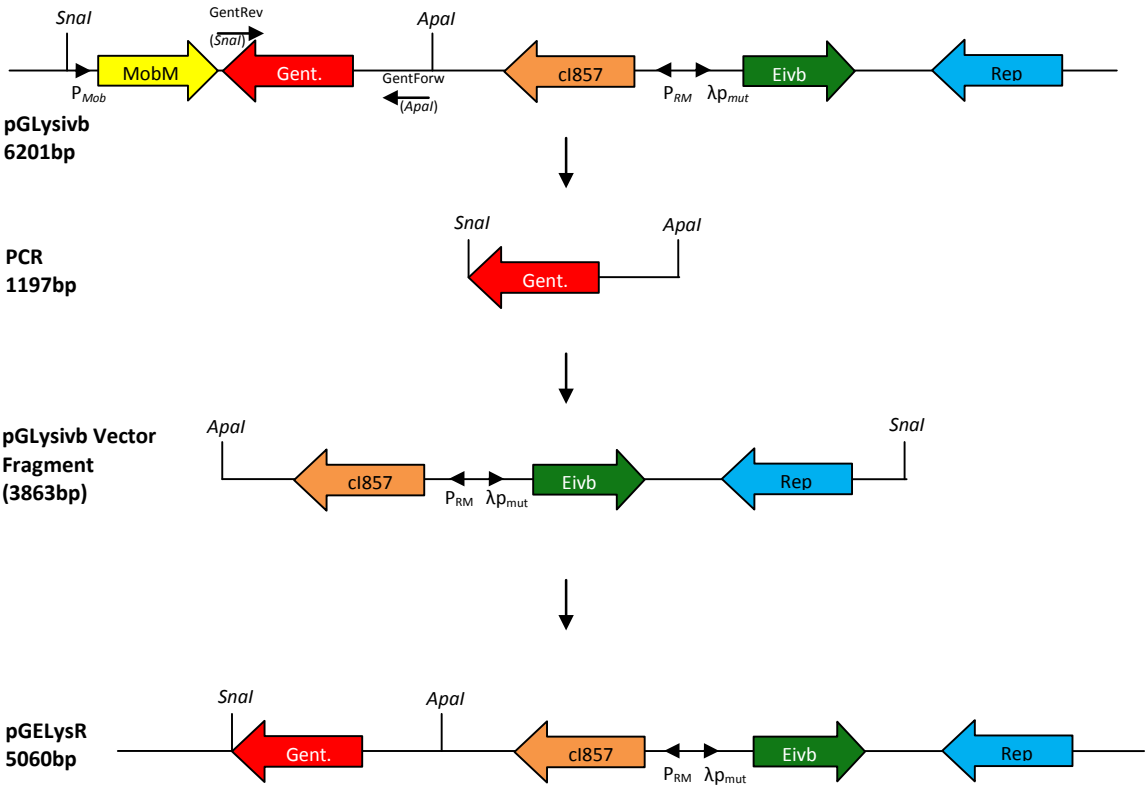


**Fig.1.** ***Cloning strategy of plasmid pGELysR;*** *$P_{Mob}$,prmoter of MobM, mutated mobilization sequence; Gent$^r$, Gentamycin resistance cassette; CI857, thermosensitive allele of the λ phage repressor gene; $P_{RM}$ / $\lambda P_{mut}$ , mutated promoter of λ phage; Eivb, in vivo biotinylated lysis protein E sequence of bacteriophage phiX174;rep, origin of replication*

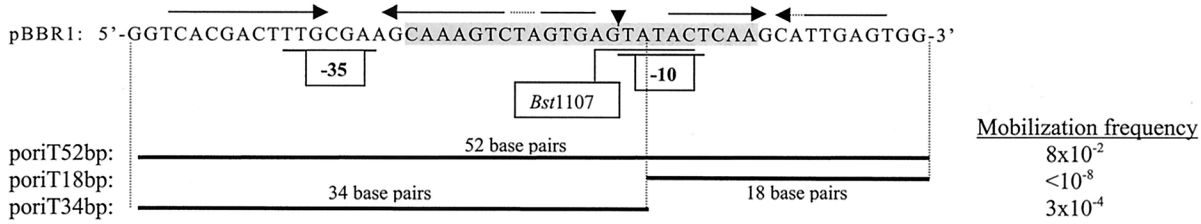


***Fig.2. Adapted from Szpirer et.al.;*** *The 52-bp sequence containing the oriT and the promoter of the mob gene. This region is present on lysis plasmid pGLysivb and its derivatives.*

## A.1.3 Results

The ligation product from above cloning procedure was transformed into the *E.coli* C2988J according to the standard molecular biological techniques by Sambrook *et.al.* [3]. About 110

bacterial colonies from 20 different transformation procedures (transformation of bacteria with this plasmid was always inefficient and only few colonies were growing on LB plates) were picked and inoculated into 5ml Luria-Bertani (LB) medium [4] supplemented with gentamycin (20µg/ml). The plasmid DNA was isolated using PeqLab Kit I (Plasmid Miniprep kit I, Erlangen, Germany). The samples were digested with restriction enzymes (purchased form Fermentas) listed in plasmid collection map and were loaded on 1% agarose gel (Roth$^{TM}$) stained using gel red nucleic acid gel stain (GelRed$^{TM}$ Biotium # 41003) and analyzed under UV light in a ChemiDOC$^{TM}$ machine (BioRad laboratories) for analyzing the right clones. A restriction digest of positive clone of plasmid pGELysR is shown in (Fig.2).

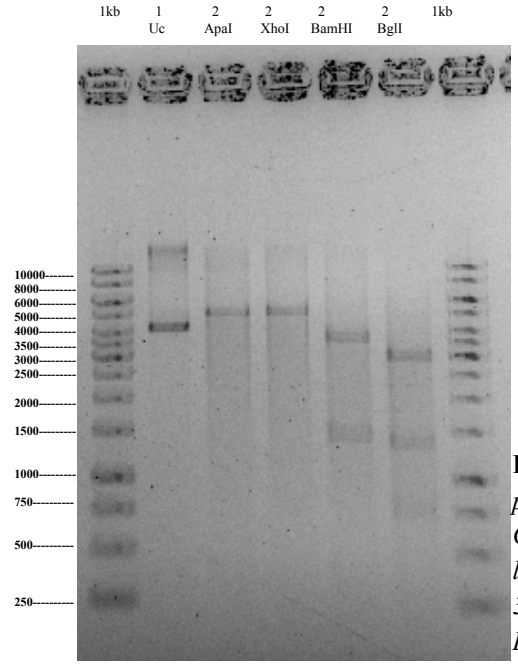


**Fig.2.** ***Restriction digest of plasmid pGELysR;*** *1% agarose gel picture showing positive clone of plasmid pGELysR ; lane1, GeneRuler$^{TM}$ 1kb DNA ladder (Fermentas); lane2, uc 5060bp; lane3, Apa*I 5060*bp; lane4, Xho*I 5060*bp; lane5, BamH*I *1433 / 3637bp; lane6, Bgl*I *739 / 1352 / 2969bp; lane7, GeneRuler$^{TM}$ 1kb DNA ladder (Fermentas).*

### A.1.4 Consistency study of plasmid pGELysR

### Procedure and results

The aim of this consistency study was to determine the lysis behavior of newly cloned plasmid. 6 single colonies of freshly transformed bacteria *E.coli* NM522 carrying plasmid pGELysR were picked and grown in 5ml LB supplemented with gentamycin (20µg/ml) and grown for several hours at 36°C and 25% glycerin stocks were prepared. Usually the consistency study is performed with three clones at a time for better and fast handling. 900µl ON culture of each clone is inoculated in a 100ml nose flask containing 25ml of fresh LB supplemented with gentamycin (20µg/ml) starting $OD_{600}$ nm of 0.1. The samples were grown

in 36°C water bath at continuous stirring 300rpm. Until $OD_{600}$ nm of 0.4-0.5 is reached. At this time lysis was induced by temperature shift from 36°C to 42°C. The lysis was continued and monitored for 2 hours through optical density observation $OD_{600}$, physical observation of bacterial cells done through light microscopy and determination of viable bacterial cells was carried out through platting dilutions of bacterial culture collected at different time points on plain LB plates using automated system spiral platter (WSAP system; DON Whitley Scientific Limited, West York Shire, UK). The LB plates were grown at 36°C over night. The colonies were counted using colony counter machine 3.15 (Synoptic Ltd., Cambridge, UK) using the program Synbiosis ProtoCOL. Six individual clones were studied during this study the average lysis efficiency was calculated to be 78.96% however best lysis efficiency was observed in clone 6 with efficiency of 93.0% and the lowest with the efficiency of 51.14% (Fig.3).

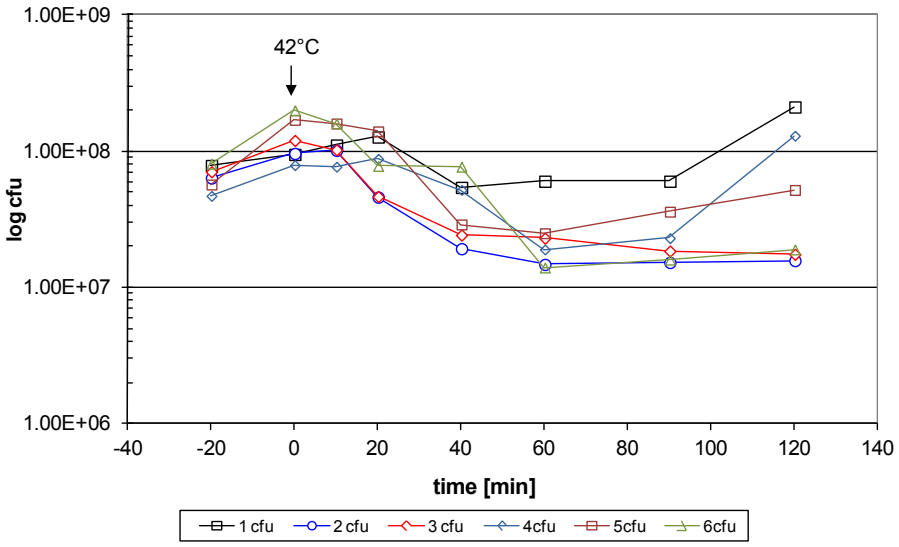


**Fig.3.** ***Consistency study of E.coli NM522 with pGELysR;*** *Cfu values of 6 different plasmid clones showing lysis efficiency of between the range of 51-93%*

### A.1.5 Discussion

Plasmid pGElysR is derivative of plasmid pGLysivb but without the mutated mobilization sequence (MobM sequence). In this study the MobM along with its promoter and *oriT* sequence was removed from plasmid pGLysivb which is a derivative of plasmid pBBR1MCS5. It is important to note that in this newly constructed plasmid the rate at which cells were growing was slower than their counter parts pGLysivb. Similarly the consistency

study showed poor lysis efficiency (Fig.3) as compared to the pGLysivb 99.762% [5]. Therefore it is more likely assumed that there are some other sequences other then *mob* and *oriT* with in this excised region who presence is crucial for plasmid stability/for its replication ability. That is why a better lysis efficiency in plasmids with MobM sequences is observed, it is shown that the lysis efficiency is directly related to the cell replication, the better cell replicates the higher is the lysis efficiency in them. However the above assumption is not proven, it needs to be studied more thoroughly before drawing any final conclusions. Therefore one must keep on searching for an alternate ways in tackling the current issue. It is shown in a study conducted by Szpirer et.al. that the *mob* gene depends on two amino acids (aspartate 120 and glutamate 121) for its mobilization thus by introducing frameshift mutation in amino acids above they managed to impair the functioning of *mob* gene by factor of $10^{-8}$ [1]. Therefore following the above mentioned strategy frameshift mutation could be introduced in the *oriT* region of the pGLysivb plasmid which has MobM gene thus making it almost impossible for current plasmid to be mobilized thus ensuring its free use in production of BGs.

## A.2 Supplementary data for chapter III

The quantitative real time PCR is the most commonly used molecular biology technique for detection and quantification of nucleic acid [6-8]. It's wide use in field of biotechnology for basic research is due to its rapid data generation, sensitivity and reproducibility [9]. The main advantage of this technique is the elimination of need to amplify and detect the final product separately on agarose gel. This technique analyzes the quantitative relationship between the amounts of target used at the start of reaction to the amount of amplified PCR product at a certain cycle. This technique is being used in quantification of minicircle DNA (mcDNA) in several studies [10, 11] and also used as a quality control criterion in production of DNA free Bacterial Ghost (BG) vaccine [12]. During this technique the SYBR Green dye is used to quantify the amount of DNA. SYBR Green dye has ability to fluorescence 100-500 times more when it intercalates with the double stranded DNA. The iCycler iQ determine the critical threshold cycle of the sample. It is the time when the fluorescence is detected statistically to be higher than the background signals. The time point at which the curves in PCR graph crosses the threshold is called quantification cycle Cq previously known as

threshold cycle $C_t$. A PCR graph generated for the quantification of mcDNA is shown in the (Fig.4).

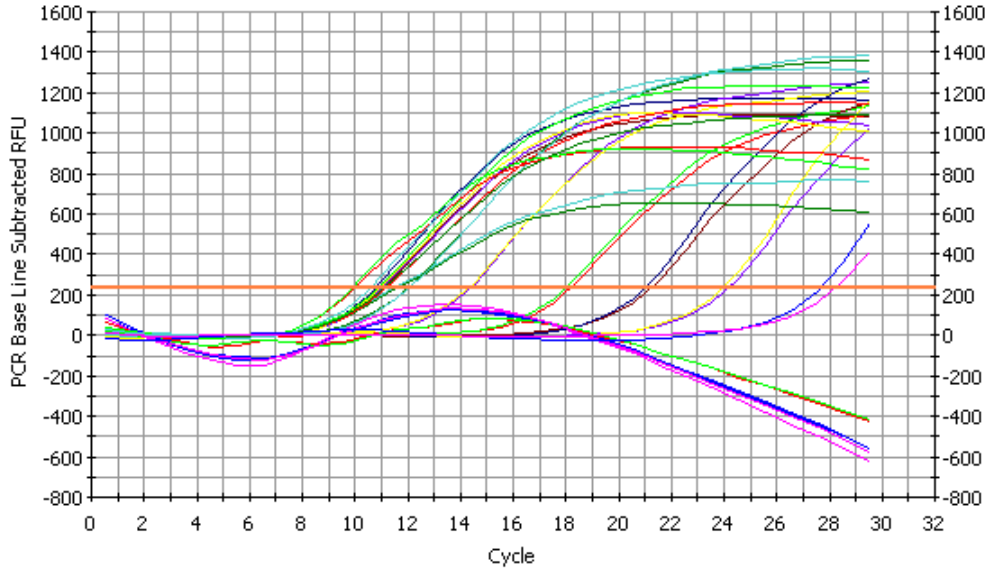


**Fig.4.** ***PCR graph;*** *showing the curves generated from mcDNA standards dilution* $10^{-1}$ *to* $10^{-6}$ *and unknown samples*

The standard curve is generated from the dilutions series constructed from the known DNA samples in this case the purified mcDNA in dilutions of $10^{-1}$ to $10^{-6}$ is used to generate the standard curve. The unknown is then plotted by the iQ software on the curve generated to quantify the amount of unknown DNA samples. The melt curve analysis for the same mcDNA can be found in chapter III under heading quantification of mcDNA and mpDNA. The standard curve generated here in this experiment shows ideal curve. The PCR efficiency shows that with every cycle the number of DNA is exponentially grown.

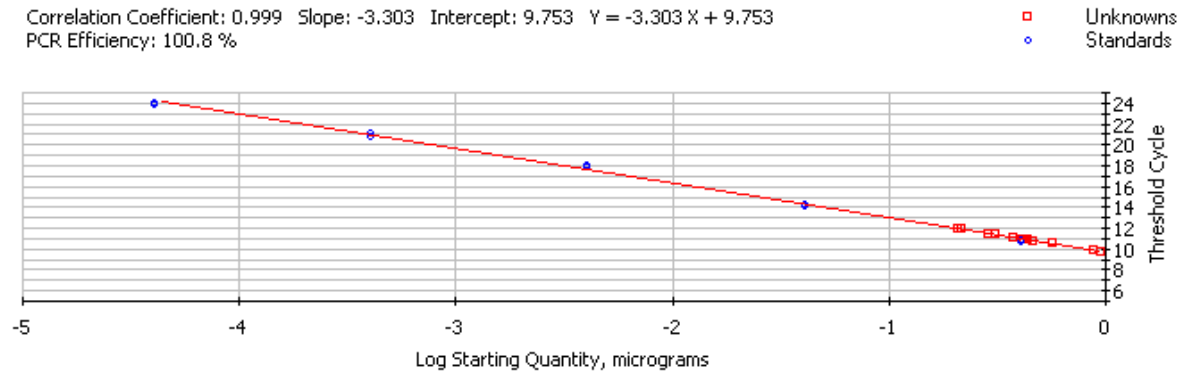


**Fig.5.** *standard curve generated by the quantification of mcDNA. 10fold serial Dilutions of purified mcDNA used to generate standard curve (blue dots); unknown samples are plotted against the standard curve (red dots)*

## A.3 Reference


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**Table.1. List of primers used**

| Template | Amplified region | Primer | Fragment size |
|---|---|---|---|
| Purified mcEYFP | 1939-1960nt<br>7805-7825nt | McXF: 5'-GTG GTT TGT CCA AAC TCA TCA A-3'<br>McSR: 5'-ACA TGA GCA GAT CCT CTA CGC-3' | 221bp |
| Purified mpDNA | 7572-7591nt Ori<br>2124-2143nt ParA | MpAF: 5'-TTT GCA AGC AGC AGA TTA CG-3'<br>MpYR: 5'-CGC AGC AGC AAA AAT AAA AG-3' | 238bp |
| mopDNA | 1939-1960nt<br>2124-2143nt ParA | McXF: 5'-GTG GTT TGT CCA AAC TCA TCA A-3'<br>MpYR: 5'-CGC AGC AGC AAA AAT AAA AG-3' | 205bp |
| pGLNIc | Gentamycin resistance gene | GentFwd1: 5'-CGATGTTACGCAGCAGGGCAG-3'<br>GentRev: 5'-CGA TGA ATG TCT TAC TAC GGA G-3' | 194bp* |
| pBHR1 | Kanamycin resistance cassette | PKanAhdIF 5'-TTA GCA *GAC GG'G GAG TC*G CCA CGT TGT GTC T -3'<br>PKanAhdIR 5'- CAC CAG *GAC GG'G GAG TC*T TAG AAA AAC TCA T -3' | 934bp |
| pSIPHCNparA | Resolution site 1 | 5'res1K 5'– CAG CAG *GGT ACC* CCT TGG TCA AAT TGG GTA TAC C –3'<br>3res1P 5' – CTG CTG *TTA TAA* GCA CAT ATG TGG GCG TGAG – 3' | 140bp |
| pEYFP-C1-MCS | CMV-EYFP | 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'<br>3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3' | 1596bp |
| pmCherry-C1-MCS | CMV-mCherry | 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'<br>3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3' | 1587bp |
| pVenusA206K-C1-MCS | CMV-Venus | 5'RYFP-SacI 5'- TGG CCA GAG CTC TAG TTA TTA ATA GTA ATC-3'<br>3'RYFP-KpnI 5'- ATA CCA GGT ACC TTA AGA TAC ATT GAT GAG -3' | 1596bp |
| pGLysivb | Gentamycin resistance | GentFowr(ApaI) 5'-ATA *GGG CC'C* GGT ACC CAG CTT TT- 3'<br>GentRev(*Sna*I) 5'- ATA *GTA'TAC* TTA GGT GGC GGT ACT TGG GTC- 3' | 1197bp |

All Primers designed using web based primer3 software using default settings
* Allaham 2006

| | |
|---|---|
| Ag | antigen; |
| APCs | antigen-presenting cells |
| BB | bacterial backbone |
| BGs | Bacterial Ghosts |
| BPL | β-propiolactone |
| CFU | colony forming units |
| CMV | cytomegalovirus |
| DCs | dendritic cells |
| EHEC | enterohemorragic *Escherichia coli* |
| EMA | European Medicines Agency |
| FDA | Food and Drug Administration |
| GnRH | gonadotrophin-releasing hormone |
| HA | heamagglutatnin |
| HBV | hepatitis B virus |
| hCG | human chorionic gonadotrophin |
| HIV | human immunodeficiency virus |
| IE | immediately early enhancer |
| IM | inner membrane |
| IPTG | isopropyl β- |
| LPS | Lipopolysaccharide |
| MBP | maltose-binding protein |
| mcDNA | minicircle DNA |
| mpDNA | miniplasmid DNA |
| mopDNA | mother plasmid DNA |
| NK cells | natural killer cells |
| NTHi | nontypable Haemophilus influenza |
| OD | optical density |
| OM | outer membrane |
| ompA | outer membrane protein A |
| PAMPs | pathogen-associated molecular patterns |
| pSIP | self-immobilizing plasmids |
| qPCR | quantitative real time PCR |
| RITARD | reversible intestinal tie adult rabbit diarrhea |
| SIP | self immobilizing plasmid |
| SNUC | *Staphylococcus aureus* nuclease A |
| TLR | toll-like receptor |
| TCP | Toxin-coregulated pilus |
| ZP | zona pellucida. |

# Curriculum vitae

## Personal Data

| | |
|---|---|
| Name: | Muhammad Abbas (Changezi) |
| Date of birth/place: | 20th December 1979/Quetta |
| Nationality: | Pakistani |
| Current address: | Guglgasse 08/1/6/22B, 1110 Vienna, Austria |

## Education and experience

| | |
|---|---|
| 2008 to present | PhD studies (Molecular Biology) at University of Vienna, Faculty of Life Sciences. Thesis: Minicircle DNA Immobilization in Bacterial Ghosts (BGs): Investigation for the Reduction of Un-recombined Mother Plasmid and Miniplasmid DNA in BGs |
| 2007-2008 | Assistant District Livestock officer, Govt. of Baluchistan, Pakistan |
| 2003-2006 | M. Phil (Molecular Biology) Centre for Excellence in Molecular Biology, University of Punjab, Pakistan. Thesis: Genetic Analysis of DFNB12 in Pakistan |
| 1998-2002 | Doctor of Veterinary Medicines (DVM) from University of Veterinary & Animal Sciences (UVAS) Lahore, Pakistan |
| 1996-1998 | Intermediate (Pre-Medical) from P.A.F. Inter College Samungli, Quetta, Pakistan |

## Scholarships and Professional Memberships

| | |
|---|---|
| 1998-2002 | Scholarship for DVM studies by Ministry of Livestock and Dairy Development Govt. of Baluchistan |
| 2008-2011 | HEC and ÖAD award for PhD studies at University of Vienna, Austria |
| 2002-present | Member Pakistan Veterinary Medical Council (PVMC) |

### Publications

Langemann, T., V. J. Koller, **A. Muhammad**, P. Kudela, U. B. Mayr, and W. Lubitz (2010), The bacterial ghost platform system - Production and applications. Bioeng Bugs 1 (5): pp.326-336.

**A. Muhammad**, J. Champeimont, U. B. Mayr, W. Lubitz and P. Kudela, Bacterial Ghosts as carriers of protein subunit and DNA encoded antigens for Vaccine applications. Expert Rev. Vaccines (Accepted to be published in Jan. 2012)